Spring 5-15-2016

Granulocyte-Colony Stimulating Factor Reprograms the Bone Marrow Microenvironment to Suppress B Lymphopoiesis

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by

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A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

May 2016

St. Louis, Missouri

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ACKNOWLEDGEMENTS

I would most like to acknowledge my advisor, Dr. Daniel Link, who has set a prime example of how to be careful, rigorous, and creative scientist. What I am most appreciative of is how seriously Dr. Link takes his responsibility as a mentor. Dr. Link makes himself available for guidance and challenges his trainees to think more deeply, propose alternate interpretations of data, and plan for the next step in an experimental program. He provides detailed feedback on any and all writing, despite the extra time it requires out of his busy schedule. He also allows his trainees freedom and flexibility to think independently, to pose new experiments or lines of research, and to make mistakes. By treating his trainees, colleagues, collaborators, and competitors with respect, he sets an example of how a good scientist not only conducts good research but also builds good relationships.

I would also like to thank the other members of the Link lab, which has been a very supportive, enjoyable, and entertaining environment to train in. Other lab members are willing to give advice or an extra pair of hands as needed, leading to a collaborative atmosphere where all members are invested in one another's' successes. Our lab managers, Jill Woloszynek and Amy Schmidt, kept the lab organized and running smoothly, and technicians Molly Romine and Fulu Liu have helped with more experiments than I can name. Jackie Tucker-Davis made my life immeasurably easier by her conscientious management of our animal facilities. Michael Zhang assisted in the analysis of the *Dmp1-Cre Cxcl12^{flox}* and *OC-Cre Cxcr4^{flox}* mice. Adam Greenbaum developed the *Cxcl12^{flox}* strain, developed much of the immunofluorescence protocol I used, and taught me many lab skills. Mahil Rao taught me how to do multicolor flow cytometry and mouse surgery, and he provided helpful advice throughout my project. Jun Xia was my mentor during my first summer rotation. My MSTP classmates, labmates, and friends, Maria

Trissal and Tim Supakorndej, have provided moral support and a sense of camaraderie during our time in lab together, as have my bay mates Bryan Anthony and Grazia Abou Ezzi. Laura Schuettpelz and Terrence Wong, oncology fellows in the lab, have provided scientific and career advice. I would also like to thank the other members of our lab, Alun Carter, Giri Ramsingh, Ghada Kunter, Yen-Michael Hsu, Kyle Eash, and Matthew Christopher, for their help and support over the years.

I would also like to acknowledge my thesis committee members: Dr. Timothy Ley, who served as chair of my thesis committee, Dr. Deepta Bhattacharya, Dr. Fanxin Long, Dr. Eugene Oltz, and Dr. Matthew Walter. All are excellent scientists with many demands on their time, and I am appreciative of the effort they have put into my project and training. The other students, faculty, and staff on the 6th floor of the Southwest Tower and the members of our Monday lab meeting (the Bessler, Schuettpelz, Dinauer, Challen, Batista, Oh, Sturgeon, and Magee labs) have provided much helpful feedback at our regular meetings. The faculty and students in the Molecular Genetics and Genomics department and the MSTP have also provided helpful feedback and advice.

The members of the Flow Cytometry Core at the Siteman Cancer Center have been invaluable for their help with my project. I would especially like to thank Bill Eades for helping me understand flow cytometry, and Jackie Hughes, Dan Schweppe, and Chris Holley for helping me with sorting experiments. Angie Schrader of the RCAVS Microscopy and Digital Imaging Core provided support for confocal imaging. The MSTP and the NHLBI/Ruth L. Kirschstein fellowship have provided funding for my research, and Brian Sullivan, Christy Durbin, Elizabeth Bayer, and Linda Perniciaro have made the machinery of the MSTP run smoothly.

I would also like to thank my previous scientific mentors. Dr. Sarah Lea Anglin at Millsaps College was my first research mentor, and I did not consider a career in research until she offered me the opportunity to work in her lab. Under her guidance I learned how much I enjoyed the act of producing new knowledge, and she put me on my current path. Dr. Loren Michel at Washington University in St. Louis offered me a job as a research technician in his lab, where he taught me many experimental techniques and gave me the opportunity to extensively participate in his research program. Dr. Guojun Bu and Dr. Joshua Rubin provided excellent guidance and advice as my rotation mentors. Finally, I would like to thank my family and friends. My MSTP classmates have been sources of fun, relaxation, friendship, and support during the years, and I am grateful to have them. I am thankful for my family, especially my parents, Susan and O.C. Day; my sisters Courtney Silewicz and Lindsay Demouy; my nieces and nephew Payten Silewicz, Ty Silewicz, and Allie Demouy; and my aunts Shelley Brent and Cheryl Maio; whose love for me has been a comfort during my life. I would especially like to thank my boyfriend, Adam Kepka, who has been loving and patient with my long hours and chaotic schedule.

ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy in Biology & Biomedical Sciences

Molecular Genetics and Genomics

Washington University in St. Louis, 2016

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The production of hematopoietic cells in the bone marrow is tightly and dynamically regulated in response to environmental stimuli. In response to infection, the bone marrow increases granulopoiesis at the expense of lymphopoiesis. The mechanisms mediating this shift are poorly understood. We show that treatment with granulocyte-colony stimulating factor (G-CSF), which is often induced during infection, results in marked decline of B lymphocytes at multiple stages of bone marrow B cell development. Transgenic mouse models show that G-CSF acts in a non-cell intrinsic fashion through cells of the monocyte-macrophage lineage to suppress B lymphopoiesis by downregulating important B trophic factors including CXCL12, KIT ligand, FLT3 ligand, interleukin-6, interleukin-7, IGF-1, and BAFF, resulting in B cell mobilization and apoptosis. G-CSF reprograms CXCL12-abundant reticular (CAR) cells, bipotent adipogenic-osteogenic stromal cells that are a key component of the B cell niche, increasing osteogenic potential and downregulating the production of CXCL12, interleukin-7, and KIT ligand. G-CSF also decreases osteoblast number, disrupting an additional B niche population and source of key B cell developmental cytokines. These data show that G-CSF reprograms bone marrow stromal

cells to alter the bone marrow microenvironment to actively suppress B lymphopoiesis. To further explore the role of stromal cell-derived CXCL12 in B lymphopoiesis, we examined mice in which *Cxcl12* was conditionally deleted in different stromal cell populations. Deletion of *Cxcl12* using *Dmp1-Cre* (targeting osteolineage cells) does not disrupt bone marrow B lymphopoiesis. Deletion of *Cxcl12* using *OC-Cre* (targeting osteolineage cells and vascular smooth muscle cells) results in an isolated loss of mature naïve B cells in the bone marrow due to a homing defect following peripheral maturation. Deletion of *Cxcl12* using *Osx-Cre* (targeting CAR cells) results in an early loss of bone marrow B cells beginning with pre-pro-B cells. Deletion of *Cxcl12* using *Prx1-Cre* (targeting mesenchymal progenitors) results in severe suppression of B lymphopoiesis, including a loss of CLP, the upstream progenitor of B, T, and NK cells. These data suggest that CXCL12 from different stromal cell populations in the bone marrow regulates distinct stages of B lymphopoiesis.

CHAPTER 1: INTRODUCTION TO THE BONE MARROW MICROENVIRONMENT AND B LYMPHOPOIESIS

The production of mature blood cells from uncommitted progenitors in the bone marrow is a tightly regulated process that is highly responsive to environmental stimuli. Hematopoietic differentiation occurs via a hierarchical pattern of lineage commitments, beginning with an uncommitted hematopoietic stem cell (HSC) with the potential to give rise to any type of hematopoietic cell. HSCs give rise to multipotent progenitors, which in turn produce lineage-restricted progenitors that ultimately give rise to mature blood cells[1]. The production of mature blood cells from progenitor populations within the bone marrow is regulated by the bone marrow microenvironment or niche, a specialized group of adjacent cells, extracellular matrix, and secreted factors that regulate proliferation and differentiation of developing cells. Different niches have been postulated to exist for different hematopoietic lineages as well as for different stages of differentiation within a single lineage[2,3]. Dysregulation of the normal maturation of hematopoietic cells in the bone marrow is associated with a variety of pathologic conditions. Better understanding how the bone marrow microenvironment regulates the proliferation and differentiation of hematopoietic cell types is therefore important to understanding how hematopoietic diseases arise and can be treated.

One of the earliest fate decisions made by an uncommitted hematopoietic progenitor is commitment to either the myeloid lineage or the lymphoid lineage. The earliest committed myeloid cell is known as the common myeloid progenitor and is capable of giving rise to red blood cells, platelets, monocytes, macrophages, osteoclasts, dendritic cells, and granulocytes[1].

The most primitive lymphoid-committed cells is known as the common lymphoid progenitor (CLP), which is capable of producing B cells, T cells, natural killer cells, and dendritic cells[4,5].

The cells of the B lineage are part of the adaptive immune system and give rise to antibody-secreting cells. B cell development occurs in both the bone marrow and peripheral lymphoid organs through a well-described stepwise progression of stages that can be identified based on surface marker expression (Figure 1.1). In the bone marrow, the CLP gives rise to the B lymphoid progenitor (BLP), the earliest known B-committed cell[6]. Pre-pro-B cells, also known as Fraction A cells, are derived from the BLP and predominately retain germline expression of immunoglobulin genes. Early pro-B/Fraction B cells have begun the process of heavy immunoglobulin chain (IgH) rearrangement, a process that is completed in late pro-B/Fraction C cells. B cells that have successfully rearranged IgH express the pre-B cell receptor (pre-BCR) on the cell surface. Signaling through the pre-BCR directs light chain recombination in pre-B/Fraction D cells. Cells that successfully rearrange light chain immunoglobulin are known as immature/Fraction E cells and express cell surface IgM, composed of a complex of rearranged heavy and light immunoglobulin chains. Most immature B cells migrate from the bone marrow to the spleen where they undergo additional maturation into mature naïve B/Fraction F cells expressing both IgM and IgD on the cell surface[7–10], though some may complete maturation in the bone marrow without transiting to the spleen[11]. Mature naïve B cells recirculate between the bone marrow and peripheral lymphoid organs. Upon exposure to a recognized antigen, they undergo additional stages of maturation in the periphery and can home back to the bone marrow as mature antibody-secreting plasma cells[9,10].

1.1. Key Cytokines Regulating Bone Marrow B Lymphopoiesis

1.1.1. CXCL12

B cell development in the bone marrow is regulated in part via soluble factors produced by the bone marrow microenvironment. CXCL12, also known as stromal-derived factor 1 (SDF-1) or pre-B growth stimulating factor, is a chemokine important for B lymphopoiesis. CXCL12 was originally isolated from stromal cell lines as a factor stimulating B cell development in *in vitro* cultures[12]. Mice deficient for CXCL12 are perinatal lethal, and fetal livers from these mice have reduced number of B cells and low CFU-pre-B activity[13]. Similarly, mice lacking the primary receptor for CXCL12, CXCR4, have an early defect in B lymphopoiesis, with a reduction in pre-pro-B cell number and reduced numbers of Lin^- IL-7Rα^+ KIT^{low} Sca^{low} cells, a compartment enriched for CLPs and BLPs[14–16]. Transgenic mice expressing an intracellular form of CXCL12, expected to inhibit CXCL12-CXCR4 signaling by sequestering CXCR4 in intracellular compartments, show decreased B cell number beginning with the pro-B stage, while overexpression of extracellular CXCL12 increases B cell number, demonstrating a requirement for extracellular CXCL12-CXCR4 signaling for normal B cell development[17].

CXCL12-deficient fetal liver cells transplanted into wild type recipients are capable of reconstituting the hematopoietic system, including B lymphopoiesis, demonstrating that CXCL12 from the non-transplantable stromal compartment, not from hematopoietic cells, is required for B cell development. Conversely, serial transplants from mice reconstituted with CXCR4-deficient fetal liver cells show reduced numbers of B cell progenitors in the bone marrow, indicating that CXCR4 signaling within B cells is required for efficient B lymphopoiesis. In these mice, the number of B cells in peripheral organs are only mildly

reduced, showing that the CXCL12-CXCR4 axis plays at most a minor role in maintenance of the peripheral B cell pool[18].

In addition to its role in enhancing B cell proliferation, CXCL12 is known to serve as potent chemoattractant for a variety of cell types, and several lines of evidence suggest that part of the B cell phenotype in CXCL12 or CXCR4 knockout mice is due to a failure of B cell trafficking. Mice deficient for either CXCL12 or CXCR4 have hypocellular bone marrow[14], suggesting a defect in the migration of hematopoietic cells from the fetal liver to the bone marrow. *Cxcr4* knockout mice have a low number of pro-B cells in the fetal liver and an elevated number in the peripheral blood, suggesting a defect in the retention of developing B cells. Transplant of CXCR4-deficient fetal liver cells into wild type mice results in a reduced percentage of B cells in the bone marrow relative to wild type controls and an increase in IgM-negative B cells detectable in the peripheral blood, indicating inefficient retention of developing precursors[19]. Use of a *Cd19-Cre* transgene to conditionally delete *Cxcr4* at the pro-B stage of development also shows a loss of bone marrow tropism in B cells, with mature B cells preferentially locating to the periphery and IgM-negative B cells detected in the spleen. Splenic IgM-negative B cells display an elevated rate of apoptosis, indicating that the bone marrow microenvironment is critical for developing B cell survival. Immunization assays show reduced homing of antigen-specific plasma cells to the bone marrow at early post-exposure time points but normal levels at 90 days, indicating that CXCL12-CXCR4 signaling plays a role in short-term plasma cell homing but redundant pathways exist for long-term homing[20]. Further evidence for the importance of the bone marrow microenvironment for developing B cell survival comes from mice deficient in the S1P1R adhesion receptor, which show a premature release of B cells from the bone marrow and increased apoptosis in the periphery[21].

Mutations in CXCR4 are known to play a role in human diseases, including diseases with perturbations in B cell development or function. WHIM syndrome is a genetic disorder named for its common clinical manifestations: warts, hypogammaglobulinemia, infections, and myelokathexis (retention of mature neutrophils in the bone marrow). Most patients with WHIM syndrome have a mutation in the carboxy-terminus of CXCR4 that results in premature truncation, believed to enhance CXCL12-CXCR4 signaling by preventing receptor internalization and downregulation[22–28]. Since CXCR4 signaling is known to promote retention of cells in the bone marrow, it has been hypothesized that hyperactive CXCR4 signaling sequesters B cells in the bone marrow, deranging normal B lymphopoiesis and resulting in hypogammaglobulinemia. B cell lymphopenia is common in patients with WHIM syndrome[29], and a mouse model in which one WHIM mutation is knocked-in to the endogenous CXCR4 locus recapitulates peripheral lymphopenia and reduced or absent B cell follicles in secondary lymphoid tissues. Bone marrow sequestration may not be the complete explanation for decreased peripheral B cells, however, since WHIM mice also show decreased pro-B/pre-B cells and immature B cells in the bone marrow, suggesting that enhanced CXCR4 signaling impairs early stages of B lymphopoiesis[30]. An activating mutation in CXCR4 found in WHIM patients is also highly recurrent in some B cell malignancies, including 28.2% of lymphoplasmacytic lymphoma cases and 20% of IgM-MGUS cases. In a murine model of lymphoplasmacytic lymphoma the mutation was found to enhance lymphoma proliferation, dissemination to the bone marrow and other organs, and chemotherapy resistance[31], indicating that CXCL12-CXCR4 signaling may be an attractive target when treating diseases featuring perturbed B cell development.

1.1.2. Interleukin-7

Interleukin-7 was originally identified as a supportive factor for B cell cultures *in vitro*[32]. Mice deficient for IL-7 have reduced B lymphocyte number in the bone marrow as well as in the peripheral blood and secondary lymphoid organs. B cells that are produced appear to have normal function, however, suggesting that IL-7 is required for efficient B cell development but may be dispensable for B cell function[33]. Consistent with the *Il7* knockout mice, mice deficient for a component of the IL-7 receptor (IL-7R), IL-7Rα, have a reduction in cellularity in lymphoid organs due to defects in B and T lymphocytes. *In vivo*, pro-B cells are the earliest stage of B lymphopoiesis where number is reduced, suggesting that IL-7 signaling is critical for the pre-pro-B to pro-B transition[34]. However, other evidence suggests that IL-7 may be important at earlier stages of B lymphopoiesis. CLPs express IL-7R but have normal numbers based on surface phenotype in both IL-7 and IL-7Rα knockout mice[35]. Upon transplant into wild type mice, however, CLPs from mice lacking IL-7 show reduced contribution to B lymphopoiesis, suggesting that IL-7 signaling at the CLP stage is required for full B cell potential[36]. Similarly, in *Il7rα* knockouts pre-pro-B cell number is normal but transcription factors important for further B cell differentiation are absent or reduced relative to wild type pre-pro-B cells[35]. Furthermore, pre-pro-B cells derived from IL-7-deficient mice are less efficient at producing B cells after transplant into wild type recipients compared to pre-pro-B cells derived from wild type animals[37], indicating IL-7 is required at early stages of B cell development in order for normal B cell differentiation to occur.

In addition to controlling B cell differentiation, IL-7 is important in the proliferation and survival of developing B cells. In *in vitro* culture, IL-7 promotes pro-B proliferation[38], and B cells derived from IL-7Rα mice fail to proliferate in *in vitro* culture[34]. IL-7R is also known to

deliver survival signals to developing B cells. IL-7 signaling plays a role in maintaining genomic integrity by inhibiting immunoglobulin rearrangement during times of B cell proliferation, preventing massive genetic instability and apoptosis as pro-B cells are rearranging immunoglobulin heavy chain[39–44]. IL-7 signaling appears to be dispensable for human B lymphopoiesis, however, since humans with mutations in the γ_c gene, an essential component of IL-7R, or *Jak3*, a downstream signaling molecule, have normal B cell number[45,46].

1.1.3. FLT3 ligand

The receptor FMS-Related Tyrosine Kinase 3 (FLT3, also known as FLK2) and its ligand FLT3L are required for normal B cell development. Mice deficient for FLT3 have normal splenic cellularity and normal myeloid development in the bone marrow but decreased bone marrow B cell number. Pre-pro-B and pro-B cell number is severely reduced, pre-B number is slightly decreased, and mature naïve B cell number is normal, indicating that FLT3 signaling is important for early stages of B cell development but non-essential for later stages. CFU-pre-B assays show decreased numbers of B cell progenitors, consistent with a defect in early B lymphopoiesis[47]. Similarly, mice deficient for FLT3L have a reduction in bone marrow cellularity due in part to decreased B cells and a reduction in CFU-pre-B in both frequency and absolute number. As in the receptor knockout, early stages of B cells in the bone marrow (pro-B and pre-B) are most severely affected, while mature B cells are less severely affected. Circulating B cells in the peripheral blood are reduced but normal in function[48]. Later studies in both *Flt3* and *Flt3l* knockout mice detected a loss of CLPs, indicating that FLT3 signaling is important in the earliest stages of lymphopoiesis. A competitive transplant where FLT3L-deficient bone marrow was competitively transplanted against wild type bone marrow showed that FLT3L knockout cells contributed equally to all lineages except B cells, suggesting that

progenitors developing in a FLT3L-deficient environment have a permanent reduction in B cell competency[49].

1.1.4. KIT ligand

KIT ligand (KITL, also known as stem cell factor) and its receptor, KIT, have been found to play a role in B cell development in adult mice. Mice with a hypomorphic *kit* allele have normal levels of mature B cells in the periphery but reductions in late pro-B, pre-B, and immature B cells in the bone marrow. The defect in B lymphopoiesis was traced back to the CLP stage[50], suggesting that KIT signaling is required for early stages of B cell development but not maintenance of mature B cell number.

1.1.5. BAFF

B cell activating factor (BAFF) and its receptor BAFF-R play a critical role in later stages of B cell development. *Baff* knockout mice show low levels of circulating immunoglobulins, small peripheral lymphoid organs, and decreased numbers of B cells in the periphery. Early stages of bone marrow B lymphopoiesis appear roughly normal, but splenic lymphopoiesis shows a block in B cell development from the immature B to mature naïve B cell stage of development. A similar defect is observed in mice lacking BAFF-R[51]. The observation that some maturation of immature B cells to mature naïve B cells occurs in the bone marrow suggests that there may be a role for BAFF in the bone marrow in addition to its role in splenic lymphopoiesis[11].

1.1.6. Interleukin-6

The cytokine interleukin-6 (IL-6) has been most extensively studied in its effects on peripheral B cell development. IL-6 is known to induce differentiation of mature B cells into plasma cells, and to enhance antibody secretion from plasma cells[52,53]. Evidence also exists that

IL-6 induces proliferation of pre-B cells *in vitro*[54], indicating that it may play a role in enhancing bone marrow B lymphopoiesis as well.

1.1.7. RANK ligand

Receptor Activator of NF-kB (RANK) and its ligand RANK ligand (RANKL) are most well-known for the role they play in osteoclast development, but they also play an important role in bone marrow B lymphopoiesis. *Rankl*$^{-/-}$ mice show decreased immature B and mature naïve B number in the spleen, but bone marrow B lymphopoiesis is difficult to assess due to extensive osteopetrosis. Transplant of RANKL-deficient hematopoietic cells into *Rag1*$^{-/-}$ mice, deficient in the production of lymphocytes, results in a block in B lymphopoiesis at the pro-B to pre-B transition[55]. Similarly, *Rank*$^{-/-}$ mice have a reduction in splenic mature naïve B cell number[56], suggesting that RANKL-RANK signaling is important for both bone marrow and peripheral B cell development.

1.1.8. Insulin-like growth factor 1

The cytokine insulin-like growth factor 1 (IGF-1) has also been reported to play a role in B lymphopoiesis. *In vitro*, addition of IGF-1 to murine B cell cultures enhances the differentiation of pro-B cells to pre-B cells, and inhibition of IGF-1 production in stromal cells impairs their ability to induce pro-B to pre-B differentiation[57]. Similar results were found in an *in vitro* assay with human cells[58]. Continuous infusion of IGF-1 into mice has also been shown to increase bone marrow B cell number[59]. Study of IGF-binding proteins (IGFBPs), carrier proteins that can enhance or inhibit IGF-1 signaling, shows that IGFPBP3 attenuates pro-B development while IGFBP-6 stimulates pro-B differentiation, providing further evidence that IGF-1 signaling can promote B cell development[58].

1.1.9. Cooperative interactions

Cooperative interactions between B trophic cytokines demonstrate that the coordinated expression of multiple cytokines in the microenvironment can play an important role in B lymphopoiesis. *In vitro* IL-7 and KITL promote CFU-pre-B colony formation more efficiently than either alone[60]. Insulin-Like Growth Factor 1 (IGF-1) is also known to synergize with IL-7 to promote B cell expansion and pre-B maturation[61]. Conversely, while mice deficient for either FLT3L or IL-7Rα have similar phenotypes, mice deficient for both have a more severe phenotype than either alone, including a more severe reduction in peripheral lymphoid organ size and an almost complete absence of functional B cells[62]. Similarly, mice deficient for both FLT3 and KIT have a more severe phenotype than either single mutant[47].

1.2. B cell niche populations

In the bone marrow, B lymphopoiesis is guided not only by secreted cytokines but also by cell-cell interactions between developing B cells and other hematopoietic or stromal populations. There are multiple lines of evidence suggesting that B cell niche populations exist and that B lymphopoiesis is altered if normal niche function is disrupted, but how niche populations regulate B cell development is unclear. The most popular model in the field is that B cells interact with different niche populations at different stages of B cell development, and that B cells move from one niche to another as they develop[63,64] (Figure 1.2). In this model, it is generally assumed that different niche cells make specific factors that are required for specific stages of B cell development. Other models are also plausible, however. It is possible that B cells remain relatively static in the bone marrow, continuously in close proximity to a small number of niche cells that provide all of the factors required for B lymphopoiesis. Such factors could be regulated temporally, with niche cells altering their transcriptional profile as B cell requirements

change, or niche cells could maintain a relatively constant production of B trophic factors, with B cell sensitivity to these secreted factors changing as B cells mature. While the niche is generally considered to consist of cells in direct contact with a developing B cell, it is possible that factors secreted by distant cells regulate some aspects of B cell development. These models are not necessarily mutually exclusive; it is possible that some elements from each model are true for specific niche populations, B cell populations, or soluble factors. Further study of the B cell microenvironment will be needed to resolve these questions.

1.2.1. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent stromal cells in the bone marrow, classically defined as having the ability to differentiate into osteoblasts, chondrocytes, and adipocytes in a fibroblastic colony forming unit (CFU-F) assay. The extent to which the CFU-F assay tests true MSC potential is unclear, since true bone marrow MSCs may give rise to more stromal cell populations than this limited set, and multipotent populations that are not MSCs may also give rise to CFU-F. *In vivo*, MSCs are considered to be rare perivascular cells, but *in situ* observation of them has been elusive, due in part to a lack of consensus on how to best identify MSCs. Various markers have been proposed to identify MSCs *in vivo* in bone marrow, including combinations of PDGFRα, PDGFRβ, CD51, and Sca; alpha smooth muscle actin; and the *Nestin-Gfp* transgene[65–69]. A lineage mapping model using *Prx1-Cre*, a transcription factor activated in limb bud mesenchyme, to activate a fluorescent reporter has also been reported to identify a rare cell highly enriched for CFU-F activity[70]. While developing B cells are not known to interact with MSCs in the bone marrow, putative MSCs have been reported to make several cytokines important in B cell development, including CXCL12, KITL, and IL-7[68,70–73].

1.2.2. CXCL12-abundant reticular cells

CXCL12-abundant reticular (CAR) cells were identified by a knock-in of *Gfp* into the *Cxcl12* locus. CAR cells are defined as CD45-negative Ter119-negative CD31-negative stromal cells expressing high levels of GFP. They have a characteristic reticular morphology, with long processes extending throughout the bone marrow, and are predominantly perivascular in location[63]. CAR cells produce high levels of CXCL12 and are considered to be the major source of CXCL12 in the bone marrow[74]. They also produce high levels of KITL[72,74].

CAR cells have been reported to be in direct contact with several hematopoietic populations, including pre-pro-B cells[63], consistent with a role for CXCL12 and KITL in early B cell development. A diphtheria toxin-ablation model points to a role for CAR cells in early stages of B cell development, since CAR cell ablation is rapidly followed by a loss of CLP and decreased proliferation and increased apoptosis of pro-B cells. HSCs also show increased commitment to the myeloid lineage, indicating that CAR cells may play a role in maintaining lymphopoietic potential. However, this model is complicated by the fact that the mice die within 5 days with severe liver necrosis[74], suggesting that CAR cell ablation has severe organism-wide effects and that the observed changes may be secondary to other effects rather than directly due to loss of CAR cells.

CAR cells were originally described as bipotent adipogenic-osteogenic cells, expressing both osteogenic and adipogenic transcription factors at the single cell level[74]. Lineage mapping using *Osterix-Cre* to target CAR cells results in fluorescent reporter activity in both CAR cells and osteoblasts, consistent with the hypothesis that CAR cells are osteoprogenitors[70]. However, *Leptin-receptor-Cre* (*Lepr-Cre*) efficiently targets CAR cells and adipocytes but not osteoblasts, casting doubt on the idea of CAR cells as osteoprogenitors[73]. This discrepancy may be resolved

by two recent papers that suggest that CAR cells give rise to osteoprogenitors in adult mice but not young mice. Primordial CAR cells are first detectable at E16.5 but express low levels of genes associated with CAR cells in adult mice[75]. *Lepr-Cre*-targeted cells, the vast majority of which are likely CAR cells, are rare at E19.5 and common in the metaphysis but not the diaphysis at P0.5, suggesting that CAR cells arise late in fetal development and are rare for much of early bone marrow development. By 2 months of age *Lepr-Cre*-targeted cells are common in both the metaphysis and diaphysis. Based on lineage mapping they have little to no contribution to chondrocytes or osteoblasts in 2 month old mice but have increasing contribution to osteoblasts with age. CFU-F assays, ossicle formation assays, sublethal irradiation, intrafemoral injection, and fracture models all provide evidence that *Lepr-Cre*-targeted cells from adult mice have osteogenic, chondrogenic, and adipogenic potential[71]. Collectively, these data suggest that *Lepr-Cre*-targeted CAR cells may contain an MSC-like population in adult mice, but that chondrocytes and osteoblasts in young mice derive from a different progenitor. Since the progenitor cell for CAR cells is currently unknown, it is also possible that *Lepr-Cre*-targeted CAR cells represent a multipotent stage in between true MSCs and more mature stromal cell populations.

Manipulation of CAR differentiation has been shown to affect B lymphopoiesis. Deletion of the *Foxc1* transcription factor in CAR cells, either constitutively or inducibly in adult mice, results in disturbed hematopoiesis, including deficits in CLP, pro-B, and pre-B cell number. Constitutive deletion results in adipocyte infiltration of the marrow, but osteoblast number and cortical thickness remain normal at the time of analysis. Inducible deletion results in a modest reduction in overall CAR cell number, increased transcription of adipocyte markers, and decreased expression of B trophic factors CXCL12 and KITL[75]. Deletion of *Pten* within CAR

cells also results in enhanced adipogenic potential at the expense of osteogenic potential, however the effect on B lymphopoiesis was not examined[71].

1.2.3. Osteoblasts/osteocytes

Osteoblasts have also been implicated in bone marrow B lymphopoiesis. Osteoblasts are known to secrete several factors important for B cell development, including IL-7, CXCL12, IGF-1, RANKL, and IL-6[76–80]. Co-culture of osteoblasts with B progenitors has shown that osteoblasts can support all stages of B cell development *in vitro*, and co-culture with uncommitted progenitors has demonstrated that osteoblastic cells can direct multipotent cells to differentiate into B cells[76]. *In vivo*, osteoblast ablation using a thymidine kinase driven by the *Col2.3* promoter has shown that ablation of osteoblasts leads to a rapid loss of B cells at early stages of B cell development[76,81]. Like other ablation models, however, this model is complicated by the possibility that loss of B cells is not directly due to loss of osteoblasts but secondary to other changes occurring in the bone marrow following widespread osteoblast apoptosis.

Other evidence pointing to an important role for osteoblasts in B cell development comes from conditional deletion models targeting osteoblasts. Conditional knockout of the $G_{\alpha}s$ signaling molecule in osteoblast precursors decreases bone marrow B cell number and increases bone marrow neutrophil number. Transplant of bone marrow from conditionally deleted mice into wild type mice rescues the loss of B cells, indicating that the defect is confined to the non-transplantable stromal compartment. Osteoblasts sorted from conditionally deleted mice have reduced levels of IL-7 mRNA, and administration of exogenous IL-7 to conditionally deleted mice partially rescues bone marrow B cell number[82].

Osteocytes embedded in bone matrix also play a role in B lymphopoiesis. Osteocytes have been reported to be an important source of RANK ligand in the bone marrow[80], which is

required for normal B cell development. Osteocytes also produce sclerostin, which promotes B cell survival, perhaps via regulation of CXCL12 production[83].

While CAR cells are one potential osteoprogenitor cell, perivascular smooth muscle cells may also be osteoprogenitors. Alpha smooth muscle actin (αSMA)-positive cells line arteries and arterioles throughout the body, including in the bone marrow. αSMA-positive cells are known to proliferate in the bone marrow after ablation of osteoblasts and to colocalize with Col3.6-GFP, a marker of early osteoblast development. αSMA-positive cells isolated from an *αSMA-Gfp* reporter mouse have the ability to give rise to osteoblastic and adipogenic cells *in vitro*, and *in vivo* can give rise to cells positive for Col2.3-CFP, a marker of mature osteoblasts, when injected intrafemorally[84]. *Ng2-Cre*, a Cre recombinase reported to target an αSMA-positive periarteriolar cell population with high level CXCL12 and SCF mRNA, has been shown to mark vascular smooth muscle cells, osteocytes, chondrocytes, and some osteoblasts in the bone marrow, but not CAR cells[71,85]. Whether CAR cells and smooth muscle cells represent two independent paths of osteogenesis or have a lineal relationship is unknown. *Lepr-Cre* has been reported to mark CAR cells but not vascular smooth muscle in the bone marrow[71], suggesting that if a lineal relationship exists, αSMA cells are progenitors of CAR cells rather than the converse. Additionally, *Osteocalcin*-Cre, a Cre recombinase that efficiently targets osteoblasts, targets some cells in the central marrow[86] that may represent an osteoprogenitor pool. It is unknown what relationship, if any, the *Osteocalcin-Cre*-targeted cells have to CAR cells.

1.2.4. IL-7-expressing stromal cells

IL-7 has been reported to be expressed by a bone marrow stromal cell distinct from CAR cells using immunohistochemical staining against IL-7[63]. Because IL-7 is produced in low quantity in the bone marrow, however, this staining has been difficult to replicate. To provide an

additional way to detect IL-7 producing cells, several groups have generated transgenic reporter mice using segments of the IL-7 promoter to drive a fluorescent reporter or Cre recombinase. Transgenic mice generated by cloning a fragment of the *Il7* promoter upstream of a fluorescent reporter or Cre recombinase inserted randomly into the genome have produced inconsistent results, with different expression patterns in mice generated by different groups, perhaps due to the different lengths of the promoter used to drive expression or due to the region of the genome in which the cassette is inserted[87–90]. Only one transgenic reporter has been reported to have bone marrow expression. In that model, *Il7* reporter activity was observed in a central marrow stromal cell that was not further characterized. Notably, no reporter activity was detected in osteoblasts. Reporter activity was not observed in other tissues known to have IL-7 expression, however, making it unclear whether the lack of expression in osteoblasts is a technical limitation of the model or if osteoblasts truly lack IL-7 expression[88]. Two knock-in models in which *Gfp* is inserted into the endogenous *Il7* locus, which should faithfully report IL-7 transcription, have recently been reported. In one model GFP expression in bone marrow stromal cell populations was noted[91], while in the other expression in the bone marrow was not described[92].

1.2.5. Galectin-1 cells

Galectin-1 is a ligand for the pre-BCR, which promotes proliferation and differentiation of pre-B cells. Staining for Galectin-1 in the bone marrow reveals that it is widely expressed, found on both hematopoietic cells and stromal cells. Within stromal cells, Galectin-1 is found on osteoblasts along the endosteum, cuboidal cells in the central marrow, and fibroblastic reticular cells in the central marrow. Adoptive transfer experiments in which late pre-B cells were labeled with a fluorescent dye and transplanted into mice treated with hydroxyurea to clear cycling cells from the marrow show that late pre-B cells preferentially interact with cuboidal Galectin-1-

expressing stromal cells in the central marrow, while pro-B and early pre-B cells do not, suggesting that Galectin-1 cells may represent a specific pre-B niche population. Use of a transgenic *Il7-Cre* reporter mouse[87] suggests that the IL-7-expressing population is distinct from the Galectin-1-expressing population; however, it is unclear if the *Il7-Cre* reporter mouse faithfully identifies IL-7 expression. Galectin-1 cells were negative for CXCL12 by qPCR, suggesting that they may also be distinct from CAR cells, though Galectin-1 expression on CAR cells themselves was not analyzed[64].

1.2.6. Dendritic cells

In addition to stromal cells, hematopoietic cells in the bone marrow also play a role in B cell development. Mature naïve B cells in the bone marrow have a perivascular location and are in close association with dendritic cells. Use of a *CD11c-Dtr* ablation model to inducibly delete dendritic cells results in a loss of mature naïve B cells but no other B cell subpopulations. The loss of mature naïve B cells is specific to the bone marrow, as mature B cell number is normal in peripheral lymphoid organs. The defect was shown to be due to decreased mature naïve B cell survival rather than a defect in homing or retention of B cells in the bone marrow[93].

1.3. G-CSF effects on the bone marrow and B cell development

1.3.1. Introduction to G-CSF

Granulocyte Colony Stimulating Factor (G-CSF) was originally identified from conditioned media as a factor able to induce differentiation of a leukemic cell line and promote the outgrowth of granulocytic colonies from murine bone marrow cultures[94]. In humans, serum levels of G-CSF rise in the acute phase of bacterial infection and decline in the recovery phase, consistent with a role for G-CSF in the early immune response[95]. Animal models provide robust evidence that G-CSF plays a key role in granulocyte development. Mice deficient for G-CSF

exhibit a peripheral neutropenia and a depletion of granulocytic precursors, demonstrating a requirement for G-CSF in baseline granulopoiesis. In a *Listeria monocytogenes* infection model, *Csf3*$^{-/-}$ mice fail to increase granulocyte production, demonstrating that G-CSF is required for emergency granulopoiesis as well[96]. Similarly, mice deficient for the G-CSF receptor (CSF3R) are neutropenic, and the neutrophils that are produced have elevated rates of apoptosis[97]. Surviving neutrophils are able to generate superoxide and degranulate normally but have impaired chemotaxis in response to multiple different stimuli due to defective adhesion and integrin signaling[98]. The ability of G-CSF to promote granulocyte development is used clinically to treat patients with deficient neutrophil number[99]. In addition to its role in granulocyte development, G-CSF treatment also results in the mobilization of HSPCs from the bone marrow into the peripheral circulation, where they can be collected and used in hematopoietic stem cell transplant[100].

1.3.2 The role of G-CSF in B lymphopoiesis

G-CSF has also been reported to affect B cell development. While the exact mechanisms by which G-CSF interacts with B lymphopoiesis are unclear, there are several lines of evidence that point to a relationship between granulopoiesis, G-CSF, and lymphopoiesis. Adjuvant-induced inflammation results in a temporary increase in bone marrow granulopoiesis at the expense of lymphopoiesis and mobilizes bone marrow lymphocytes into the periphery. In this model, developing granulocytes and lymphocytes were observed to occupy similar sites in the bone marrow, suggesting that competition for resources may lead to a reciprocal relationship[101,102]. Implantation of the CE murine tumor line into a wild type mouse induces a peripheral neutrophilia and depletion of bone marrow lymphocytes. This phenotype is recapitulated with the injection of conditioned media from CE cells grown *in vitro*, and a

candidate approach identified G-CSF as the factor responsible for both peripheral neutrophilia and medullary lymphopenia[103]. Transgenic mice overexpressing G-CSF show a reduction in bone marrow B lymphocytes and CFU-pre-B colonies[104], while mice deficient for G-CSF or CSF3R show the opposite phenotype, with increased B lymphocytes and CFU-pre-B in the bone marrow[96,105].

G-CSF is known to affect multiple stromal cell populations in the bone marrow. G-CSF treatment induces osteoblast apoptosis[106], consistent with reports that transgenic mice overexpressing G-CSF and human receiving chronic G-CSF treatment develop osteoporosis[107–109]. G-CSF treatment downregulates osteoblastic genes as well as B-trophic factors CXCL12, IL-7, and KITL mRNA in *Nestin-Gfp+* stromal cells[68]. G-CSF treatment also increases CFU-F number in the bone marrow and increases the osteogenic commitment of CFU-F cells[106,110], perhaps to compensate for G-CSF-induced osteoblast apoptosis.

The mechanisms by which G-CSF inhibits B lymphopoiesis are largely unknown. G-CSF is known to work through both direct and indirect mechanisms. CSF3R is expressed on a variety of hematopoietic cells, and the defect in granulopoiesis in *Csf3r* knockout mice is primarily due to a cell intrinsic defect in granulocyte progenitors[105]. It is unclear if CSF3R is expressed on B cells, as some groups report CSF3R expression on some populations of bone marrow and peripheral blood B lymphocytes, while others report expression only on myeloid-lineage cells[111,112]. G-CSF is also known to affect other cell populations via an indirect mechanism. Chimeric mouse models demonstrate that CSF3R expression is required on a cell in the transplantable compartment, but not HSPCs themselves, in order for G-CSF-induced HSPC mobilization to occur[113]. Similarly, G-CSF signaling through an intermediary in the hematopoietic compartment is sufficient to induce osteoblast apoptosis[106]. Evidence from

multiple groups working independently suggests that G-CSF signaling in cells of the monocyte-macrophage lineage is responsible for mediating these effects[114–116].

Recent evidence shows that G-CSF results in the mobilization of B cells from the bone marrow and B cell apoptosis. Transgenic expression of the anti-apoptotic protein BCL2 partially rescues this phenotype, though this evidence is complicated by the fact that the transgenic line utilized expresses BCL2 in all hematopoietic cells, not just B cells, making it unclear if B cell apoptosis is a direct or indirect consequence of G-CSF-treatment. It is currently unknown if the G-CSF-induced loss of bone marrow B lymphocytes is merely a side effect of increased granulopoiesis displacing developing B lymphocytes, or if G-CSF actively suppress B lymphopoiesis by altering the bone marrow microenvironment. G-CSF may signal directly through B cells to induce apoptosis, decrease proliferation, alter lineage commitment of B progenitors, or mobilize B cells to peripheral organs, or indirectly alter B lymphopoiesis by regulating the bone marrow microenvironment in a manner less permissive of B cell development.

1.4. The role of the niche in B-ALL

The bone marrow microenvironment is known to play an important role in B cell-acute lymphoblastic leukemia (B-ALL). Despite having high rates of response to induction chemotherapy, adult B-ALL has a poor overall prognosis, with a 5 year survival rate of approximately 10% for patients over the age of 60[117,118]. The poor survival rate is believed to be due in part to outgrowth of minimal residual disease consisting of chemotherapy-resistant leukemic cells. Work from several groups has produced evidence that the B cell niche in the bone marrow may play a role in protecting leukemic blasts from the full effect of chemotherapy, allowing subpopulations of blasts to acquire additional mutations leading to chemoresistance.

CXCL12-CXCR4 signaling plays an important role in B-ALL engraftment in the bone marrow, and the CXCR4 antagonist/partial agonist AMD3100 has been shown to synergize with chemotherapy in mouse models of ALL[119,120]. *In vitro*, co-culture of B-ALL tumor cell lines with stromal cells results in decreased rates of B-ALL apoptosis, both at baseline and in response to chemotherapy[121]. *In vivo*, B-ALL cell lines engrafted into mice show increased quiescence within certain bone marrow microenvironments, and neutralization of niche factors synergizes with chemotherapy to increase survival[122]. Better understanding how the microenvironment regulates B cell development, and how G-CSF shapes that regulation, may provide insights that can be applied to treating human B-ALL.

1.5. Summary

B lymphopoiesis in the bone marrow is regulated by the B cell niche, consisting of stromal cells, hematopoietic cells, and secreted factors that coordinate B cell proliferation and differentiation, and disruption of the niche can impair normal B cell production. How different niche populations regulate developing B cells is largely unknown, however. Administration of G-CSF also disrupts B lymphopoiesis, but the mechanisms mediating this disruption are unclear. Better understanding how niche populations support B cell development, and how G-CSF impairs B cell development, will lead to a better understanding of how bone marrow niches regulate B lymphopoiesis and how niche regulation may be exploited to treat B cell malignancies.

In Chapter 2, the mechanisms underlying G-CSF-mediated B cell suppression will be explored. In Chapter 3, the role of CXCL12 from different stromal cell populations in regulating B cell development will be dissected. Finally, in Chapter 4, these findings on niche regulation of B lymphopoiesis will be summarized and future directions will be outlined.

1.6 Figures

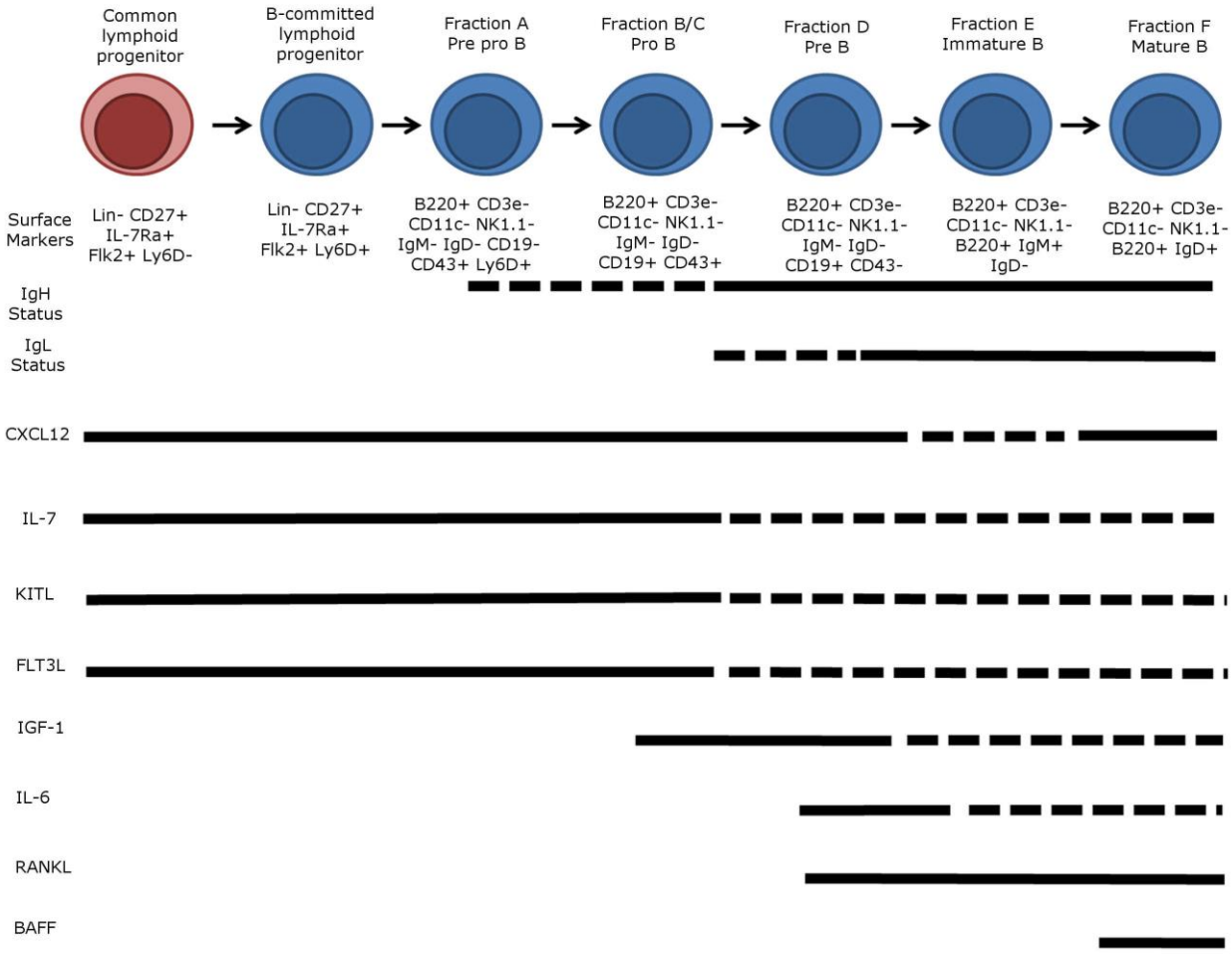

Figure 1.1. Overview of bone marrow B cell development. Shown are the surface markers, immunoglobulin recombination status, and cytokine responsiveness of different stages of bone marrow B cells.

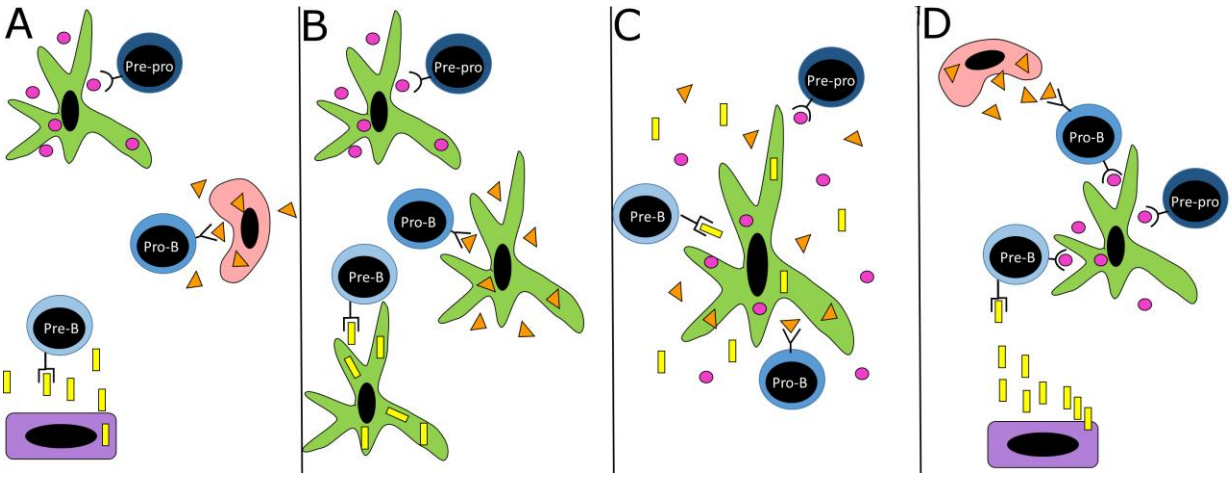

Figure 1.2. Cartoon of different niche models. **A.** Different niche cells produce factors required for distinct stages of B lymphopoiesis, and B cells at different developmental stages migrate to different niche cells. **B.** A dominant niche cell type is capable of producing factors required for different stages of B lymphopoiesis, producing specific cytokines appropriate for the developmental stage of B cells it is in contact with. **C.** A dominant niche cell type constitutively produces B trophic factors, while regulation of receptor expression on developing B cells restricts signaling to the appropriate developmental stage. **D.** Developing B cells are in contact with one type of niche cell but also receive trophic support from distant support cells.

CHAPTER 2: G-CSF REPROGRAMS STROMAL CELLS TO ACTIVELY SUPPRESS B LYMPHOPOIESIS

2.1. INTRODUCTION

The production of hematopoietic cells in the bone marrow is a tightly regulated process that is highly responsive to environmental stimuli. In response to acute infectious stress, there is a shift in hematopoiesis in the bone marrow from lymphopoiesis to granulopoiesis. Granulocyte-colony stimulating factor (G-CSF) is often induced during the acute phase of bacterial infection[95], and it has been established as the key regulator of both steady-state and stress granulopoiesis[96,97]. G-CSF has also been shown to decrease bone marrow B cell number, but the mechanisms by which G-CSF targets B cells in the bone marrow are largely unknown.

B lymphopoiesis is dependent on the production of supportive signals by bone marrow stromal cells. Moreover, there is evidence that different stages of B cell development are supported by distinct stromal cell populations, suggesting the presence of several lymphoid niches in the bone marrow. Pre-pro B cells are in direct contact CAR cells, perivascular stromal cells with osteogenic and adipogenic potential[63,71,74]. CAR cells produce high level CXCL12 and KIT ligand[72,74], important factors for B cell development, and conditional ablation of CAR cells or deletion of *Cxcl12* from CAR cells results in the loss of early B cell precursors[70,71]. Deletion of the transcription factor *Foxc1* in CAR cells results in increased adipocytic differentiation, decreased production of CXCL12 and KIT ligand, and decreased B cell number[75], indicating that alterations in CAR cell differentiation can also modulate hematopoiesis. IL-7-expressing cells are in contact with pro-B cells[63], while Galectin-1 cells have been reported to be in contact with later pre-B cells[64]. Osteoblasts and osteocytes are known to produce a number of factors important for developing B cells, including IGF-1, IL-6, IL-7, RANK ligand, and CXCL12[76–80],

and ablation of osteoblasts is also known to impair B lymphopoiesis[76,81]. G-CSF is known to induce osteoblast apoptosis[106], but its effect on other stromal cells is unclear. Understanding how G-CSF regulates B-supportive stromal cells in the bone marrow may lead to a better understanding of how stromal cells and hematopoietic cells regulate one another. Because malignant cells are believed to co-opt the stromal microenvironment for engraftment and survival signals, stromal cells may be able to be targeted to treat bone marrow malignancies.

2.2 MATERIALS AND METHODS

Mice. All mice were backcrossed at least 10 generations and maintained on a C57BL/6 background with the exception of *Cxcl12^{gfp}* mice. *Cxcl12^{gfp}* mice were a gift from Takashi Nagasawa (Kyoto University, Japan), and *Col2.3-Gfp* mice were a gift from David Rowe (University of Connecticut). *E-mu-Bcl2* mice were a gift from Barry Sleckman. The generation of *CD68:Csf3r Csf3r$^{-/-}$* mice has been previously described[116]. Mice were maintained under standard pathogen free conditions according to methods approved by the Washington University Animal Studies Committee. G-CSF was obtained from Amgen.

Blood, bone marrow, spleen, and lymph node analysis. Blood, bone marrow, spleen, and lymph node cells were harvested using standard techniques and quantified using a Hemavet automated cell counter (CDC Technologies) or Cellometer (Nexelcom).

Generation of bone marrow chimeras. Bone marrow from *Csf3r$^{-/-}$* mice expressing *Ly5.2* was mixed at a 2:1 ratio with wild-type marrow expressing *Ly5.1/5.2* and transplanted retro-orbitally into lethally irradiated *Ly5.1* recipients. A total of 3×10^6 cells were injected per recipient mouse. Recipient mice were conditioned with 1100 cGy from a ^{137}Cesium source at a rate of

approximately 95 cGy/minute before transplantation. Prophylactic antibiotics (trimethoprim-sulfamethoxazole; Alpharma, East Bridgewater, NJ) were given during the initial 2 weeks after transplantation. Mice were analyzed 8 to 10 weeks after transplantation.

Flow cytometry. Flow cytometry data were collected on a Gallios 10-color, 3-laser flow cytometer (Beckman Coulter) and analyzed with FlowJo software (Treestar). Cells were stained by standard protocols with the following antibodies (eBiosciences unless otherwise noted): Ly5.1 (A20, CD45.1), Ly5.2 (104, CD45.2), Ly6C/G (RB6-8C5, Gr-1), CD3e (145-2C11), CD45R (RA3-6B2, B220), CD11c (N418), TER-119, Flk2 (A2F10), (LG.7F9), IL-7Ra (gift of Deepta Bhattacharya, Washington University), Ly6D (49-H4, BD Biosciences), CD11c (N418), NK1.1 (PK136), IgM (II/4), IgD (11-26c), PE-Cy7-conjugated CD19 (eBio1D3), CD43 (S7, BD Biosciences), CD45 (30-F11), CD31 (390), and CD11b (M1/70).

For cell cycle analysis, cells were fixed using the Cytofix/Cytoperm kit (BD Biosciences). After fixation, cells were stained with Ki-67 (B56, BD Biosciences) and resuspended in 1 mg/mL of 4',6-diamidino-2-phenylindole (DAPI) in PermWash buffer. Doublets were excluded by FSC vs. FSC-W gating. Fractions A-D were combined into one group as $B220^{+}$ $CD3e^{-}$ $CD11c^{-}$ $NK1.1^{-}$ IgM^{-} IgD^{-} or $B220^{+}$ IgM^{-} IgD^{-}.

For apoptosis analysis, macrophages were depleted by incubating flushed bone marrow cells in sterile cell culture dishes for 1 hour. Non-adherent cells were transferred to new dishes for an additional 3 hours. Cells were washed and stained for surface markers using standard protocols, then stained for Annexin V (BD Biosciences, eBioscience) and resuspended in 1 mg/ml DAPI.

Stromal cell analysis and sorting. To extract bone marrow stromal cells, femurs and tibias were crushed with a mortar and pestle in phosphate buffered saline (PBS). Cells in suspension were collected and stored on ice while bone chips and aggregates were digested using collagenase type II (3mg/mL, Worthington Biochemical) and dispase (4mg/mL, Roche) at 37°C for 1 hour in a shaking water bath. Following digestion both fractions were pooled, red blood cells lysed, and analyzed. Cells were stained with 7-amino-actinomycin D (7AAD) to exclude dead cells and autofluorescent debris. CAR cells were defined as $CD45^-$ $TER119^-$ $CD31^-$ GFP^{bright} cells in mice carrying the *Cxcl12*^{gfp} transgene. Osteoblasts were defined as $CD45^-$ $TER119^-$ $CD31^-$ GFP^+ cells in mice carrying the *Col2.3-Gfp* transgene. Cell sorting was performed on Reflection (iCyt) or Synergy (Sony) cytometers.

Immunostaining of bone sections. Femurs and tibias were harvested and fixed for 16-24 hours in 4% paraformaldehyde (Sigma-Aldrich) at 4ºC. Bones were washed twice in PBS, decalcified in 14% ethylenediaminetetraacetic acid (EDTA) pH 7.4 solution for 3-5 days, and cryoprotected in 30% sucrose in PBS for 16-24 hours. Bones were then snap frozen in OCT media (Tissue-Tek), and tissue blocks were sectioned (10 µm or 100 µm) using the CryoJane (Leica Biosystems). Antibodies used included anti-mouse CD45R (RA3-6B2, B220) and anti-mouse IgM (II/4). Sections were washed in TNT buffer (0.1 M Tris-HCl, 0.15 M NaCl, 0.05% Tween-20) and mounted with Prolong Gold Antifade Reagent with DAPI (Invitrogen). Slides were imaged using a LSM 700 confocal microscope and ZEN imaging software (Zeiss). Volocity image processing software (Perkin Elmer) was used to calculate distances between cells.

Quantitative RT-PCR. For total bone marrow RNA, femurs or tibias were flushed with 1 mL of Trizol (Invitrogen) and RNA was prepared according to manufacturer's instructions. cDNA was prepared using SuperScript III (Invitrogen) or Taqman RT Enzyme (Applied Biosystems). qRT-PCR was performed using the TaqMan Universal RT Master Mix (Applied Biosystems) using no template and no RT controls. Data was collected on a 7300 Real-Time PCR System (Applied Biosystems). Primers were: CXCL12 forward, 5'-GAGCCAACGTCAAGCATCTG-3'; CXCL12 reverse, 5'-CGGGTCAATGCACACTTGTC-3'; CXCL12 dT-FAM/TAMRA probe, 5'-TCCAAACTGTGCCCTTCAGATTGTTGC-3'; β-actin forward, 5'-ACCAACTGGGACGATATGGAGAAGA-3'; β-actin primer; β-actin dT-VIC/TAMRA probe, 5′-AGCCATGTACGTAGCCATCCAGGCTG-3′. IL-6, KIT ligand, FLT3L, BAFF, IGF-1, and RANKL primers and probes were purchased from Applied Biosystems. IL-7 qPCR was performed using SYBR Green and the following primers: IL-7 forward, 5′-TCTGCTGCCTGTCACATCATC; IL-7 reverse, 5′-GGACATTGAATTCTTCACTGATATTCA, or primers from Applied Biosystems.

RNA expression profiling. Lineage$^-$ CD45$^-$ GFP^{bright} CAR cells from *Cxcl12^{gfp}* mice or lineage$^-$ CD45$^-$ GFP$^+$ osteoblasts from *Col2.3-Gfp* mice were sorted directly into lysis buffer, and RNA was prepared using the RNA XS column kit (Macherey-Nagel, Bethlehem, PA) according to the manufacturer's directions. RNA was amplified using the NuGen Ovation system (NuGen, San Carlos, CA), and hybridized to the Affymetrix MoGene 1.0 ST array. Data normalization was performed using the Robust Multichip Average (RMA) algorithm. Submission of this RNA expression data to Gene Expression Omnibus is in progress.

CFU-F assay. Bone marrow cells were harvest by standard techniques. For CFU-F, cells were plated in αMEM with 10% lot-tested fetal calf serum. Cells were left undisturbed for 1 week, then media was subsequently changed every 3-4 days. Alkaline phosphatase staining was performed at 2 weeks using the Leukocyte Alkaline Phosphatase Kit (Sigma Aldrich). For osteoblastic culture conditions, CFU-F medium was supplemented with 10 mM β-gylcerophosphate (Sigma Aldrich) and 50 ug/ml ascorbic acid. Cells were left undisturbed for 1 week, and media was subsequently changed every 3-4 days. Von Kossa staining was performed after 3 weeks of culture using 2.5% silver nitrate (Sigma Aldrich).

Statistics. Significance was determined using Prism software (GraphPad). Unless otherwise stated, statistical significance of differences was calculated for two groups using Students t-test and 3 or more groups using 1- or 2-way ANOVA. P-values less than 0.05 were considered significant. All data are presented as mean ± SEM. The RNA expression profiling data was analyzed using Statistical Analysis of Microarrays (SAM). A FDR < 0.05 was considered significant.

2.3. RESULTS

2.3.1. G-CSF suppresses bone marrow B lymphopoiesis at all stages of bone marrow B cell development

To examine the effect of G-CSF on bone marrow B lymphopoiesis, wild-type mice were treated with G-CSF for 5 days and lymphocyte number quantified by flow cytometry. Consistent with previous reports[103,123], total B cell number was markedly reduced (8.1 ± 0.9-fold) compared with control mice (Figure 2.1B), while neutrophil number, as expected, was significantly increased (Figure 2.1A). Likewise, G-CSF treatment was associated with significant reductions

in T cells, natural killer cells, and CD11c+ dendritic cells in the bone marrow (Figure 2.1C). Interestingly, the loss of B cells in the bone marrow was delayed, becoming significant after 3 days of G-CSF, reaching a nadir after 7 days of G-CSF, and returning to near normal levels 7 days after stopping G-CSF (Figure 2.1D). Bone marrow B lymphopoiesis proceeds through a well-described sequence of steps, beginning with a common lymphoid progenitor (CLP) with B, T, NK, and dendritic cell potential to mature naïve recirculating B cells. To determine which stages of B cell development are affected by G-CSF treatment, we measured these cell populations in the bone marrow via flow cytometry (Figure 2.2A & 2.2B). Surprisingly, although all lymphoid lineages were suppressed, no decrease in CLP number was observed after G-CSF treatment. Instead, G-CSF treatment resulted in a progressive loss of B cells at discrete stages of development. A modest loss of BLP and pre-pro-B cells suggests an early defect in B lymphopoiesis. A further decrease in B cells was observed in the transition from pre-pro-B to pro-B cells. Finally, a severe reduction in mature naïve B cells was observed, suggesting a further defect in B lymphopoiesis between immature B and mature B cells (Figure 2.2C). These data show that G-CSF treatment disrupts bone marrow B lymphopoiesis at multiple discrete stages of development.

2.3.2. G-CSF treatment is associated with increased cycling and apoptosis of certain B cell subsets in the bone marrow

We next examined the effect of G-CSF on B cell proliferation and apoptosis. We focused our analysis on days 3-4 of G-CSF, the time at which B cell number in the bone marrow most rapidly decreases. In control mice, there was a progressive increase in quiescence from early B cell precursors (Fraction A-D) to mature B cells (Fraction F) (Figure 2.3A and 2.3B). G-

CSF treatment was associated with a significant increase in cycling cells at all measured stages of B cell development, and was most striking in Fraction A-E cells. Thus, the loss of B cells in the bone marrow is not caused by decreased B cell proliferation. Apoptosis was assessed in B cells by measuring cell surface expression of Annexin V (Figure 2.3.C). In the bone marrow, we detected a significant increase in apoptosis in mature naïve B cells but not in early B precursors or immature B cells (Figure 2.3D).

2.3.3. G-CSF treatment is associated with peripheral apoptosis of developing B cells

G-CSF is known to be a potent mobilizer of neutrophils and hematopoietic stem cells from the bone marrow. However, no significant increase in total B cell number in the peripheral blood, spleen, or lymph nodes was observed after G-CSF treatment (Figure 2.4A). Thus, the loss of B cells in the bone marrow induced by G-CSF is not secondary to their bulk mobilization into the peripheral circulation. We next examined B cell subpopulations in the spleen following G-CSF treatment. While overall B cell number and mature B cell number in the spleen were not increased, a significant increase in certain precursor B cell populations was observed after G-CSF treatment (Figure 2.4B). To determine if B cell precursors mobilized to the spleen undergo elevated rates of apoptosis, we performed Annexin V staining. Regardless of G-CSF treatment, compared with the bone marrow, a much higher percentage of Fraction A-D cells in the spleen were apoptotic (Figure 2.4C), indicating that the spleen does not provide a supportive environment for early B cell development. Consistent with this hypothesis, mice carrying the *E-mu-Bcl2* transgene, expressing anti-apoptotic BCL2 in B cells, show an accumulation of early B precursors in the spleen after G-CSF treatment (Figure 2.4D) similar to a recent report using

Vav-Bcl2[123]. These data suggest that early B cell precursors mobilized to the spleen are destined for rapid clearance.

2.3.4. Mature naïve B cells do not home to the bone marrow of G-CSF-treated recipients

Despite seeing elevated Annexin V staining on mature B cells in the bone marrow of G-CSF-treated mice, we did not observe a significant rescue of mature B cell number in the bone marrow of *E-mu-Bcl2* mice treated with G-CSF (Figure 2.5A). Because mature naïve B cells home to the bone marrow following peripheral maturation, it is possible that surviving mature naïve B cells do not home or are not retained in the bone marrow of G-CSF-treated mice. To test this hypothesis we performed an adoptive transfer experiment in which splenocytes from a CAG-GFP mouse, ubiquitously expressing GFP, were adoptively transferred into PBS or G-CSF-treated recipients. 24 hours after transfer recipients were sacrificed and the number of GFP^+ IgD^+ cells in the bone marrow was quantified by flow cytometry (Figure 2.5B). We found a significant reduction in the number of GFP^+ IgD^+ cells in the bone marrow, suggesting that mature naïve B cells fail to home or be retained in the bone marrow of G-CSF-treated mice (Figure 2.5C). These results indicate that increased survival alone may not be sufficient to rescue mature B cell number in the bone marrow. Consistent with this hypothesis, we observed increased mature B cell number in the spleen of *E-mu-Bcl2* mice treated with G-CSF (Figure 2.5D).

2.3.5. G-CSF signals through monocyte-macrophage lineage cells to downregulate B trophic factors in the bone marrow

The G-CSF receptor (CSF3R) is expressed on a subset of multipotent progenitors and B cells[111,124]. Thus, it is possible that G-CSF acts in a cell intrinsic fashion to suppress B

lymphopoiesis. Alternatively, G-CSF treatment also alters the bone marrow microenvironment[79,106,113], raising the possibility of a non-cell autonomous mechanism. To address this question, we generated *Csf3r*$^{-/-}$ (G-CSF-receptor deficient) mixed bone marrow chimeras by transplanting a mixture of wild-type and *Csf3r*$^{-/-}$ bone marrow cells into lethally irradiated wild-type recipients. After engraftment, mice were either left untreated or treated with G-CSF for 5 days (Figure 2.6A). Following G-CSF treatment, wild-type B cells in the bone marrow were reduced 10-fold compared to untreated mice. Importantly, a similar decrease in *Csf3r*$^{-/-}$ B cells was observed (12-fold decrease compared with untreated mice, Figure 2.6B). In contrast, G-CSF is known to act in a cell intrinsic fashion to stimulate granulopoiesis[105]. Accordingly, G-CSF treatment of the mixed chimeras resulted in an expansion of only *Csf3r*$^{+/+}$ neutrophils (Figure 2.6C). Together, these data show that G-CSF acts in a non-cell intrinsic fashion to suppress B lymphopoiesis.

Since G-CSF acts non-cell intrinsically to suppress bone marrow B cell development, we examined the bone marrow microenvironment for potential candidates mediating G-CSF-induced B cell suppression. Bone marrow stromal cells are known to produce a number of trophic factors important for B cell development, including CXCL12, IL-7, interleukin-6 (IL-6), FLT3 ligand, KIT ligand, insulin-like growth factor 1 (IGF-1), RANK ligand (RANKL), and B cell activating factor (BAFF). We measured the mRNA expression of these genes in the bone marrow by flushing femurs with Trizol. While RANKL mRNA expression was not significantly altered, significant decreases in CXCL12 (5.8-fold), IL-6 (4.3-fold), IL-7 (7.4-fold), KITL (5.5-fold), FLT3L (2.2-fold), IGF-1 (7.9-fold), and BAFF (9.7-fold) mRNA in the bone marrow were observed with G-CSF treatment (Figure 2.6D).

G-CSF works through cells of the monocyte-macrophage lineage to mobilize hematopoietic stem and progenitor cells from the bone marrow[114–116]. To determine if monocyte-macrophage lineage cells were also responsible for mediating G-CSF-induced B cell suppression, we used *CD68:Csf3r Csf3r*$^{-/-}$ transgenic mice in which the G-CSF receptor is expressed only on monocyte-macrophage lineage cells[116]. After 5 days of G-CSF treatment, bone marrow B cell number was significantly decreased relative to PBS-treated mice (Figure 2.7A). As in wild type mice treated with G-CSF, we also observed mobilization of B precursors to the spleen (Figure 2.7B) and downregulation of B trophic factors in the bone marrow (Figure 2.7C) in *CD68:Csf3r Csf3r*$^{-/-}$ mice. Collectively, these data show that G-CSF signaling in monocyte-macrophage cells results in the downregulation of B trophic factors in the bone marrow microenvironment, promoting B cell mobilization and apoptosis.

2.3.6. G-CSF reprograms CAR cells towards an osteoblastic fate

CAR cells are mesenchymal progenitors that represent an important component of the bone marrow lymphoid niche[63,74], and alterations in CAR cell potential have been reported to affect the ability of CAR cells to support B lymphopoiesis[75]. To determine if G-CSF targets CAR cells, we used transgenic mice carrying a knock-in of the green fluorescent protein gene (*Gfp)* into the *Cxcl12* locus that identifies CXCL12 expressing cells, including GFP^{hi} CAR cells[63]. Surprisingly, CAR cell number increased after G-CSF treatment (Figure 2.8A), but we detected no change in CAR cell morphology or reticular processes (Figure 2.8B). CAR cells are mesenchymal progenitors with adipogenic-osteogenic lineage potential. RNA expression profiling of sorted CAR cells showed that G-CSF treatment significantly suppressed genes associated with adipocyte differentiation, including PPARγ, lipoprotein lipase, and adiponectin,

but not osteoblast differentiation (Figure 2.8C & 2.8D, Table 2.1). Gene set enrichment analysis indicated a relative loss of adipogenesis in G-CSF-treated CAR cells (Figure 2.8E). To assess lineage commitment of CAR cells, colony-forming unit-fibroblast (CFU-F) assays were performed. CAR cells are the major source of CFU-F in the bone marrow[71], and consistent with an increase in CAR cell number (Figure 2.8A), G-CSF treatment results in an increase in CFU-F (Figure 2.9A). Osteogenic potential was assessed by alkaline phosphatase staining of CFU-F. Whereas, alkaline-phosphatase positive CFU-F were significantly increased with G-CSF treatment, no change in alkaline phosphatase-negative CFU-F were observed (Figure 2.9B). Consistent with this finding, culture of CFU-F under osteogenic conditions showed a 4.74 ± 2.98-fold increase in CFU-OB (Figure 2.9C). Collectively, these data suggest that G-CSF treatment support the osteogenic potential of CAR cells, while suppressing adipogenic potential.

2.3.7. G-CSF suppresses CAR cell expression of B trophic factors

CAR cells produce many of the B trophic factors suppressed by G-CSF. RNA expression profiling of sorted CAR cells after G-CSF treatment revealed that CAR cells constitutively express CXCL12, IL-7, Kit ligand, IGF-1, and FLT3 ligand, and expression of all of these genes, except FLT3 ligand, was decreased following G-CSF treatment (Figure 2.10A & 2.10B, Table 2.1). CAR cells also express Galectin-1, associated with a stromal B niche population[64], though expression is not significantly altered with G-CSF treatment. Similarly, we observe a non-significant reduction in the expression of FOXC1, a transcription factor whose expression is correlated with CAR support of B lymphopoiesis[75]. Consistent with prior reports, we show that B cell precursors in the bone marrow localize near CAR cells (Figure 2.10C). After G-CSF

treatment, although the number of B cell precursors is markedly reduced, the remaining cells remain in close proximity to CAR cells (Figure 2.10D).

2.3.7. G-CSF suppresses osteoblasts and osteoblast expression of B trophic factors

Osteoblasts also are an important component of the bone marrow lymphoid niche[76,81,82]. As reported previously, G-CSF treatment results in a loss of mature osteoblasts in the bone marrow[106], as assessed by flow cytometry for Col2.3-GFP$^+$ stromal cells (Figure 2.11A). We next sorted osteoblast lineage cells using the Col2.3-GFP transgenic mice. Similar to CAR cells, G-CSF resulted in a marked suppression of several key B trophic factors, including CXCL12, IL-7, and KIT ligand (Figure 2.11B).

2.4. DISCUSSION

G-CSF has previously been reported to be the major cytokine regulating the shift from bone marrow lymphopoiesis to granulopoiesis in response to infectious stress[96,97,103,105]. In the present study, we show that G-CSF-induced B cell suppression is not a passive process due to myeloid expansion or peripheral mobilization of lymphocytes, but instead an active process mediated by alterations in the bone marrow microenvironment. Despite the dramatic loss of B cells, T cells, NK cells, and dendritic cells, phenotypic CLP number in the bone marrow is normal, suggesting that G-CSF does not affect commitment of hematopoietic stem cells to the lymphoid lineage. Our data show that G-CSF suppresses B lymphopoiesis at multiple discrete stages of development, including: 1) production and/or maintenance of BLP; 2) the transition of pre-pro-B cells to pro-B cells; and 3) maintenance of mature B cells in the bone marrow. Our data suggest that the loss of mature B cells in the bone marrow is due, at least in part, to increased apoptosis,

consistent with a previous report indicating that loss of dendritic cells leads to decreased mature B survival[93]. Our data also indicate that G-CSF results in impaired homing or retention of mature B cells in the bone marrow, suggesting that defective B cell trafficking may contribute to the loss of mature B cells. The decrease in B cell precursors is due, again at least in part, to their mobilization to the spleen where they undergo high rates of apoptosis. Indeed, a previous study showed that the spleen does not provide a supportive microenvironment for early stages of B cell development[20]. Our data are broadly consistent with a study by Winkler et al, which showed that enforced expression of BCL2 partially rescued the G-CSF-induced loss of bone marrow B cells[123]. While both studies noted a loss of bone marrow B cells at early stages of development, in contrast to that study we did not observe a rescue of pre-pro-B cells in the bone marrow. We also observed an accumulation of early B precursors in the spleen of *E-mu-Bcl2* mice treated with G-CSF relative to PBS-treated *E-mu-Bcl2 mice* or wild type G-CSF-treated mice, while in their study *Vav-Bcl2* mice had similarly elevated numbers of B precursors in the spleen whether treated with PBS or G-CSF. These discrepancies may be due to the different *Bcl2* transgenes used, since *E-mu-Bcl2* is restricted to B lineage cells while *Vav-Bcl2* is expressed in all hematopoietic cells, suggesting that G-CSF-induced apoptosis of other hematopoietic cell types may play a role the impairment of B lymphopoiesis by G-CSF.

Surprisingly, we observed that G-CSF treatment is associated with increased proliferation of B cell precursors. Proliferation of developing B cells in the bone marrow is tightly regulated to allow for rearrangement of immunoglobulin chains in non-dividing cells, since rearrangement within dividing cells can lead to genetic instability, cell death, or leukemic transformation[39,41,42,125,126]. Of note, IL-7 negatively regulates immunoglobulin rearrangement by suppressing expression of recombination activating gene-1 (RAG1)[40,43,44]. Thus, G-CSF-

induced suppression of IL-7 expression in the bone marrow may promote immunoglobulin gene rearrangement in proliferating B cells, leading to increased cell death.

Stromal cells are physically associated with hematopoietic stem cells, lymphoid and myeloid progenitors, and lineage committed hematopoietic populations in the bone marrow where they provide supportive and regulatory signals in specialized niches[63,64,68,70,72–75,93,127–130]. Under steady state conditions, niche signals support a balance of lymphopoiesis and myelopoiesis. We show that G-CSF specifically targets stromal cells in lymphoid niches to actively suppress lymphopoiesis. Of note, G-CSF does not directly target stromal cells; instead, it directly targets monocytic cells in the bone marrow that, in turn, regulate stromal cell function. G-CSF has been reported to utilize a similar mechanism to induce hematopoietic stem and progenitor cell mobilization by our and other groups[114–116].

G-CSF works in part by targeting CAR cells, a multipotent stromal cell population that gives rise to bone marrow adipocytes and osteoblasts[71,74]. CAR cells are known to be in contact with pre-pro-B cells[63], and ablation of CAR cells results in apoptosis of pro-B cells[74]. Recent work from two independent groups has focused on manipulation of CAR cell differentiation. Deletion of *Pten* in CAR cells results in enhanced adipocyte potential and downregulation of the osteogenic transcription factor Runx2[71]. Likewise, deletion of the *Foxc1* transcription factor in CAR cells results in enhanced expression of adipogenic genes, increased adipogenesis, decreased transcription of CXCL12 and KIT ligand, and decreased ability to support B lymphopoiesis[75]. To our knowledge, ours is the first study to report the ability of an exogenous factor, G-CSF, to influence CAR cell differentiation *in vivo*. Our findings that G-CSF increases CAR cell number and osteogenic potential are consistent with previous reports that CAR cells contain most of the CFU-F activity in the bone marrow[71] and that G-CSF increases CFU-F number in the bone

marrow[106,110]. G-CSF treatment results in decreased CAR cell expression of important B trophic factors including CXCL12, IL-7, IGF-1, and KIT ligand. Interestingly, while *Foxc1* deletion is associated with increased adipogenesis, decreased CXCL12, decreased KIT ligand, and decreased ability to support B lymphopoiesis[75], G-CSF treatment impairs B lymphopoiesis while decreasing adipogenic potential in CAR cells, suggesting that osteogenic potential in CAR cells does not serve as a surrogate for B niche function. As reported previously, G-CSF treatment results in a modest decrease in mature osteoblasts[106], which have been implicated in B cell homeostasis[76,81,82]. It also suppresses osteoblast production of B trophic factors, including CXCL12, IL-7, and KIT ligand. Of note, a previous study showed that G-CSF also targets *Nestin-GFP*$^{+}$ stromal cells, altering their expression of certain HSC maintenance genes[68]. Thus, G-CSF treatment appears to broadly affect stromal cells comprising lymphoid and stem cell niches. Since it is often induced in response to infectious stress, G-CSF provides a mechanism to broadly shape hematopoiesis through regulation of the bone marrow microenvironment.

Collectively, these data show that G-CSF actively suppresses lymphopoiesis by targeting stromal cells that contribute to lymphoid niches in the bone marrow. G-CSF reprograms CAR cells, increasing osteogenic potential, and dramatically inhibits the expression of a number of B trophic factors in CAR cells and osteoblasts, resulting in impaired B lymphopoiesis at multiple stages of B cell development. These data raise the possibility that G-CSF treatment, by disrupting supportive lymphoid niche signals, may render malignant lymphoid cells more sensitive to chemotherapy. A clinical trial in patients with relapsed or refractory acute lymphoblastic leukemia is underway to test this hypothesis (clinicaltrials.gov NCT01331590).

2.5 ACKNOWLEDGEMENTS

We thank Jill Woloszynek, Amy Schmidt, Fulu Liu, Molly Romine, and Olivia Sutton for technical assistance, Jackie Tucker-Davis for animal care, William Eades, Jackie Hughes, and Dan Schweppe for cell sorting assistance, Dr. Barry Sleckman for the *E-mu Bcl2* mice, and Dr. David Rowe for the *Col2.3-Gfp* mice. Microscopy imaging support was provided by Angie Schrader and the Microscopy and Digital Imaging Core of the Research Center for Auditory and Vestibular Studies, NIH P30DC004665. This work was supported by NIH grants RO1 HL60772 (DCL) and F30 HL112552-02 (RBD).

2.6 AUTHOR CONTRIBUTIONS

RBD designed and performed the research, analyzed the data, and wrote the manuscript. DB provided technical assistance and assisted with data analysis. TN provided the *Cxcl12*^{gfp} mice. DCL supervised all of the research and edited the manuscript, which was approved by all co-authors. The authors have no conflicts of interest to disclose.

2.7 FIGURES

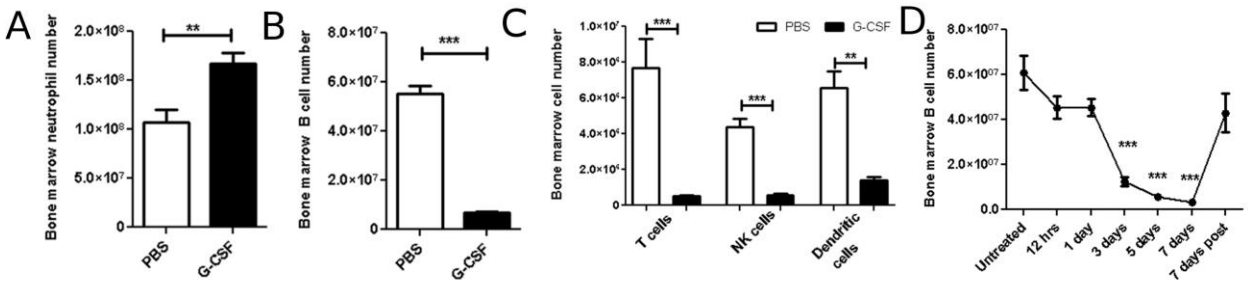

Figure 2.1. G-CSF treatment suppresses bone marrow B lymphopoiesis. **A-C.** Wild type C57BL/6 mice were treated for 5 days with 250 ug/kg G-CSF. Show is the number of bone marrow neutrophils (**A**), B220+ B cells (**B**), T cells, NK cells, or dendritic cells (**C**). **D.** Mice were treated with G-CSF for the number of days indicated and the number of B cells in the bone marrow was quantified. Data represent the mean ± SEM of 3-12 mice. *P < 0.05; **P < 0.01; ***P < 0.001; ns: non-significant.

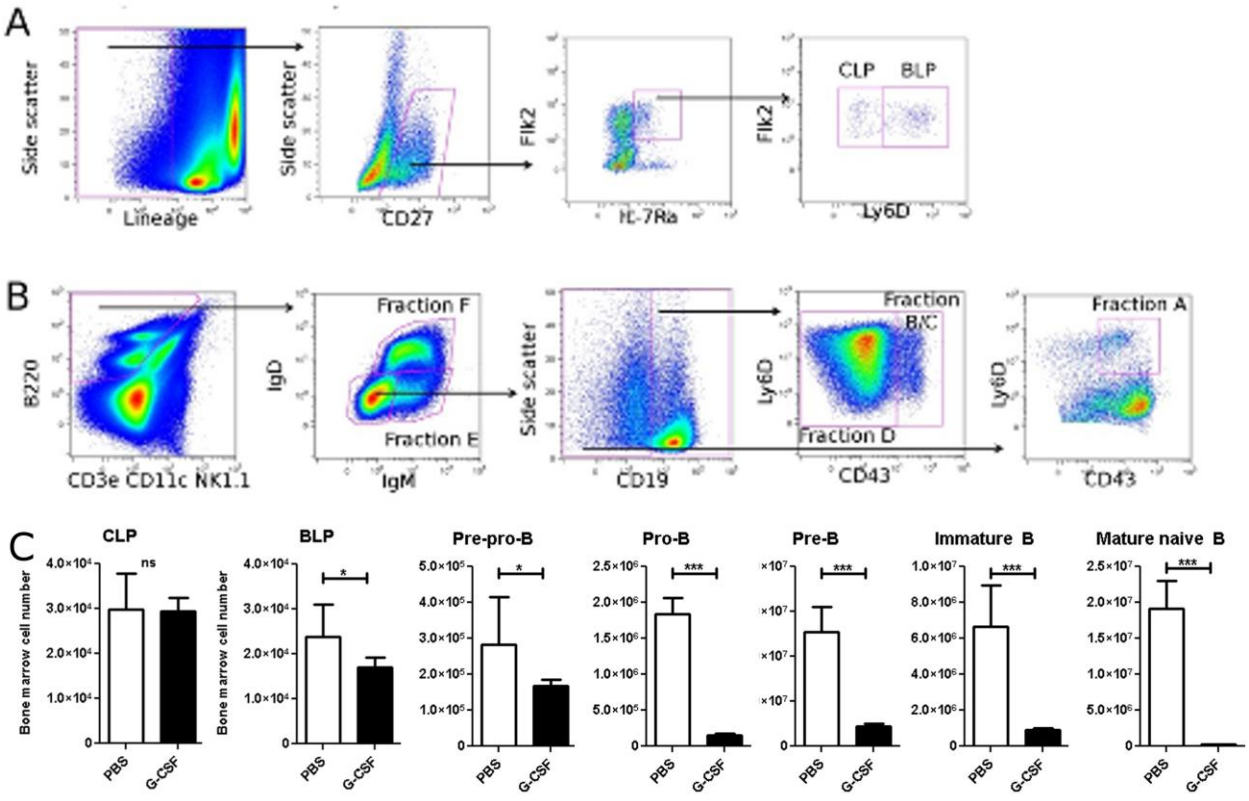

Figure 2.2. G-CSF treatment suppresses all stages of bone marrow B cell development. **A & B.** Shown is the gating strategy used for CLP and BLP (**A**) or pre-pro-B, pro-B, pre-B, immature B, and mature naïve B (**B**). **C.** Mice were treated with 250 ug/kg G-CSF for 5 days and the number of B cell subpopulations was enumerated by flow cytometry. Data represent the mean ± SEM of 10-12 mice. *P < 0.05; **P < 0.01; ***P < 0.001; ns: non-significant.

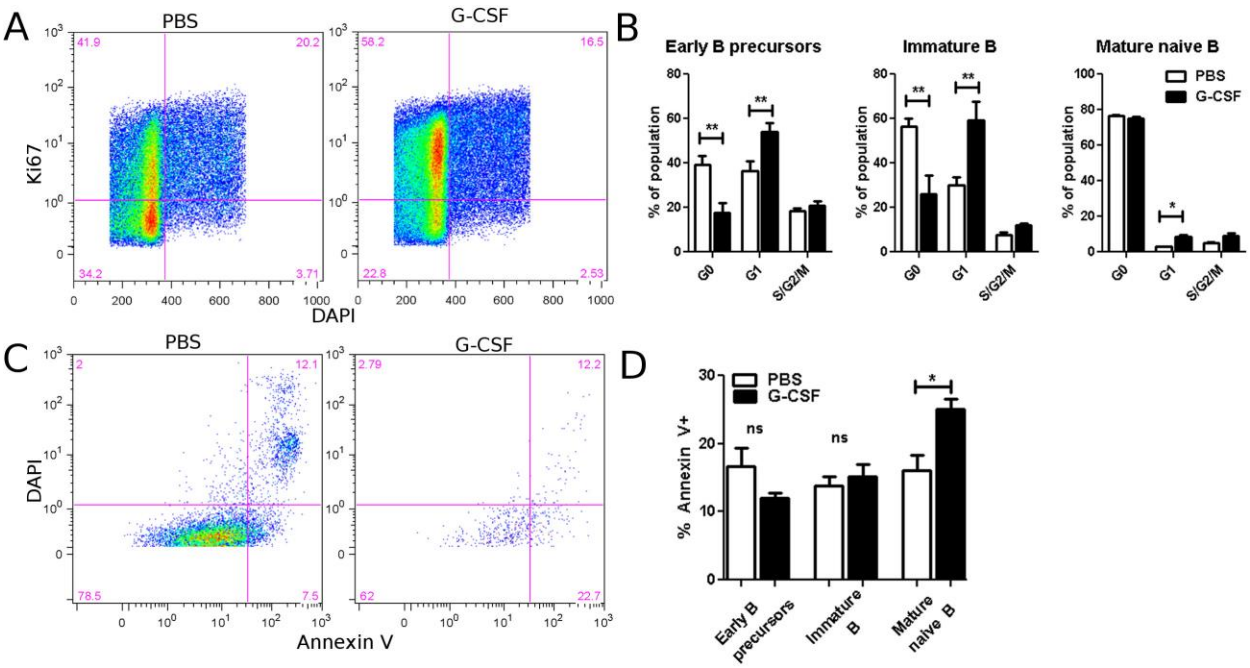

Figure 2.3. G-CSF treatment is associated with increased cycling and apoptosis in the bone marrow. **A.** Representative flow plots showing the gating scheme used for cell cycle analysis. **B.** The percentage of cells in the indicated phase of the cell cycle is shown. **C.** Representative flow plots showing the gating scheme used for Annexin V staining. **D.** The percentage of Annexin V^+ cells in the indicated B cell subset in the bone marrow is shown. Data represent the mean ± SEM of 3-9 mice. *P < 0.05; **P < 0.01; ***P < 0.001; ns: non-significant.

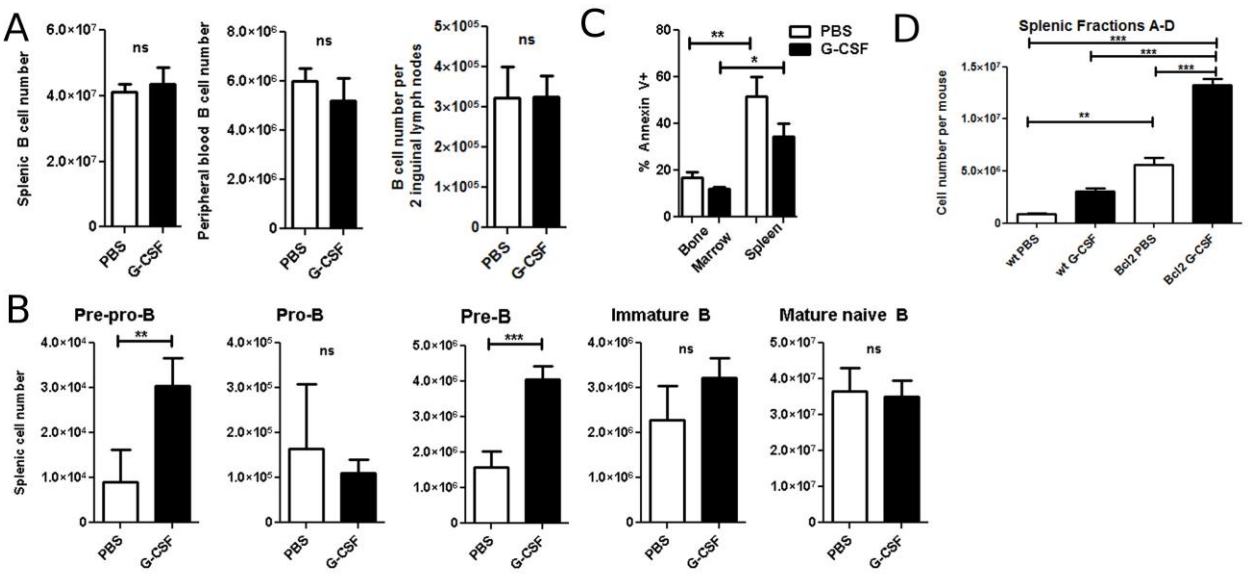

Figure 2.4. G-CSF treatment is associated with B cell progenitor mobilization and apoptosis.

A. Shown is the number of $B220^+$ B cells in spleen (left), peripheral blood (center), or lymph nodes (right) in PBS or G-CSF-treated mice. **B.** The number of the indicated B cell subset in the spleen on day 5 of G-CSF or saline treatment is shown. **C.** The percentage of Annexin V^+ cells in Fractions A-D cells in the spleen and bone marrow is shown. **D.** The number of B precursors in the spleen of wild type or *E-mu Bcl2* mice treated with PBS or G-CSF. Data represent the mean ± SEM of 5-14 mice. *P < 0.05; **P < 0.01; ***P < 0.001; ns: non-significant.

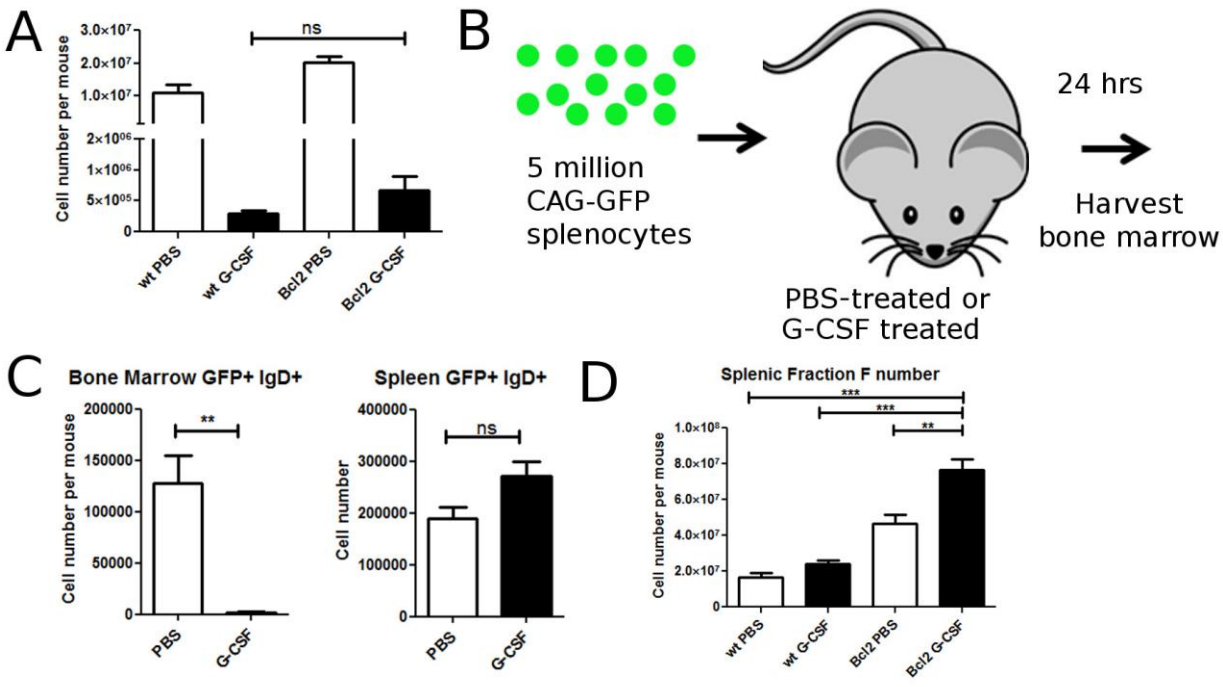

Figure 2.5. Mature B cells fail to home to the bone marrow of G-CSF-treated recipients. **A.** The number of mature B cells in the bone marrow of wild type or *E-mu Bcl2* mice treated with G-CSF or PBS. **B.** CAG-GFP splenocytes were injected into the peripheral blood of PBS or G-CSF treated mice. 24 hours after injection bone marrow was harvested. **C.** The number of GFP^+ IgD^+ mature B cells in the bone marrow (left panel) or spleen (right panel) 24 hours after transfer. **D.** The number of mature B cells in the spleen of wild type or *E-mu-Bcl2* mice treated with G-CSF. Data represent the mean ± SEM of 5-14 mice. *P < 0.05; **P < 0.01; ***P < 0.001; ns: non-significant.

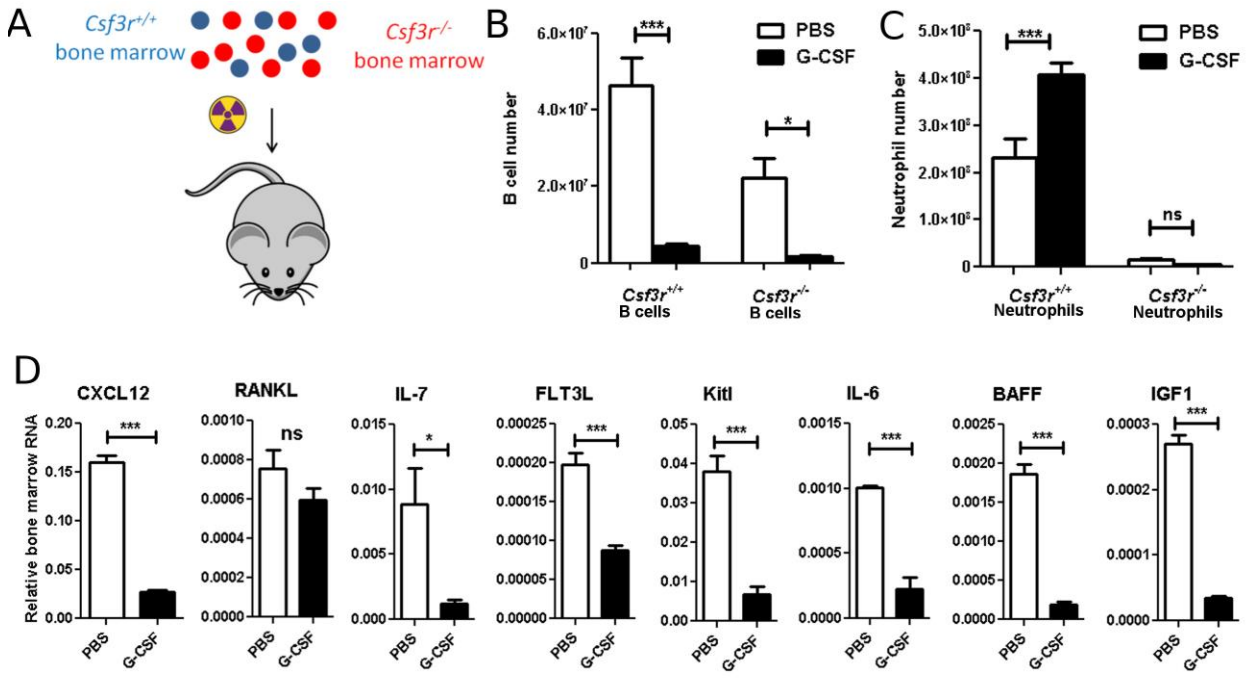

Figure 2.6. G-CSF acts in a non-cell autonomous fashion to suppress B lymphopoiesis. **A.** Mixed chimera mice were generating by transplanting wild-type (Ly5.1/5.2) and *Csf3r*$^{-/-}$ (Ly5.2) bone marrow cells into irradiated wild-type (Ly5.1) recipients at a 1:2 ratio. Eight weeks after transplantation mice were treated with G-CSF for five days or left untreated, and B cell number was analyzed by flow cytometry. **B & C**. Shown is the number of wild-type (Ly5.1^{+}/Ly5.2^{+}) or *Csf3r*$^{-/-}$ (Ly5.2^{+}) B cells (**B**) or neutrophils (**C**) in the bone marrow of G-CSF treated or untreated mice. **D**. Wild-type mice were treated with G-CSF or saline (PBS) for five days, and femurs directly flushed with Trizol to collect total bone marrow RNA. Shown is the mRNA expression of the indicated gene relative to β-actin mRNA. Data represent the mean ± SEM of 4-5 mice. *P < 0.05; **P < 0.01; ***P < 0.001; ns: non-significant.

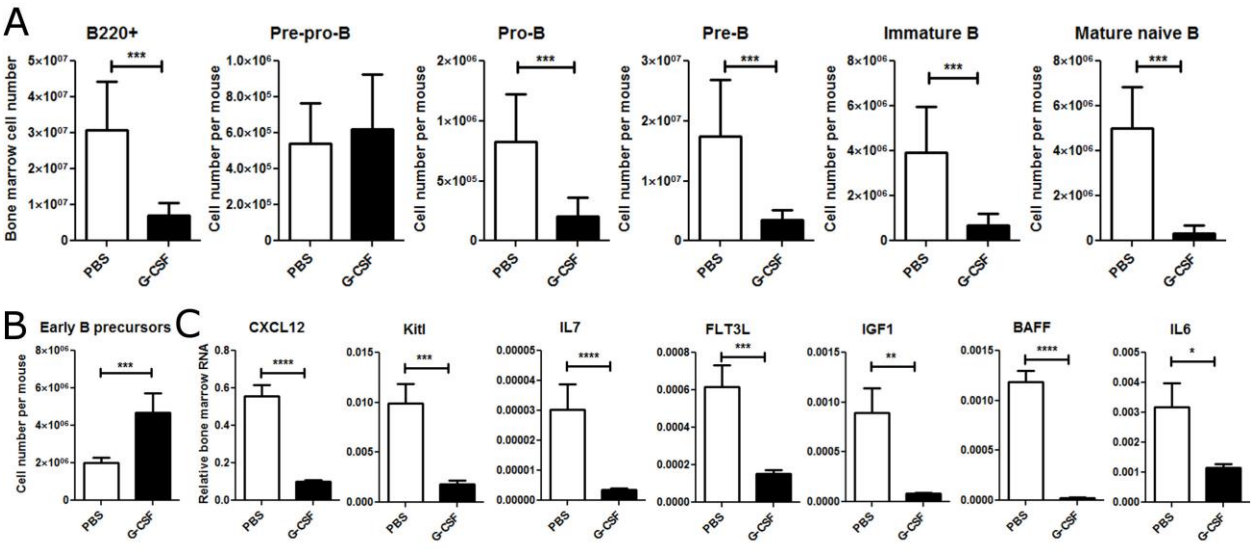

Figure 2.7. G-CSF works through cells of the monocyte-macrophage lineage to suppress B lymphopoiesis. **A.** *CD68:Csf3r Csf3r*$^{-/-}$ mice were treated for 5 days with PBS or G-CSF. Shown is the number of B cells in the bone marrow for each B cell subset. **B**. Shown is early B cell precursors in the spleen of *CD68:Csf3r Csf3r*$^{-/-}$ mice treated with PBS or G-CSF. **C**. *CD68:Csf3r Csf3r*$^{-/-}$ femurs were flushed with Trizol to collect total bone marrow RNA. Shown is the mRNA expression of the indicated gene relative to β-actin mRNA. Data represent the mean ± SEM of 7-8 mice. *P < 0.05; **P < 0.01; ***P < 0.001; ns: non-significant.

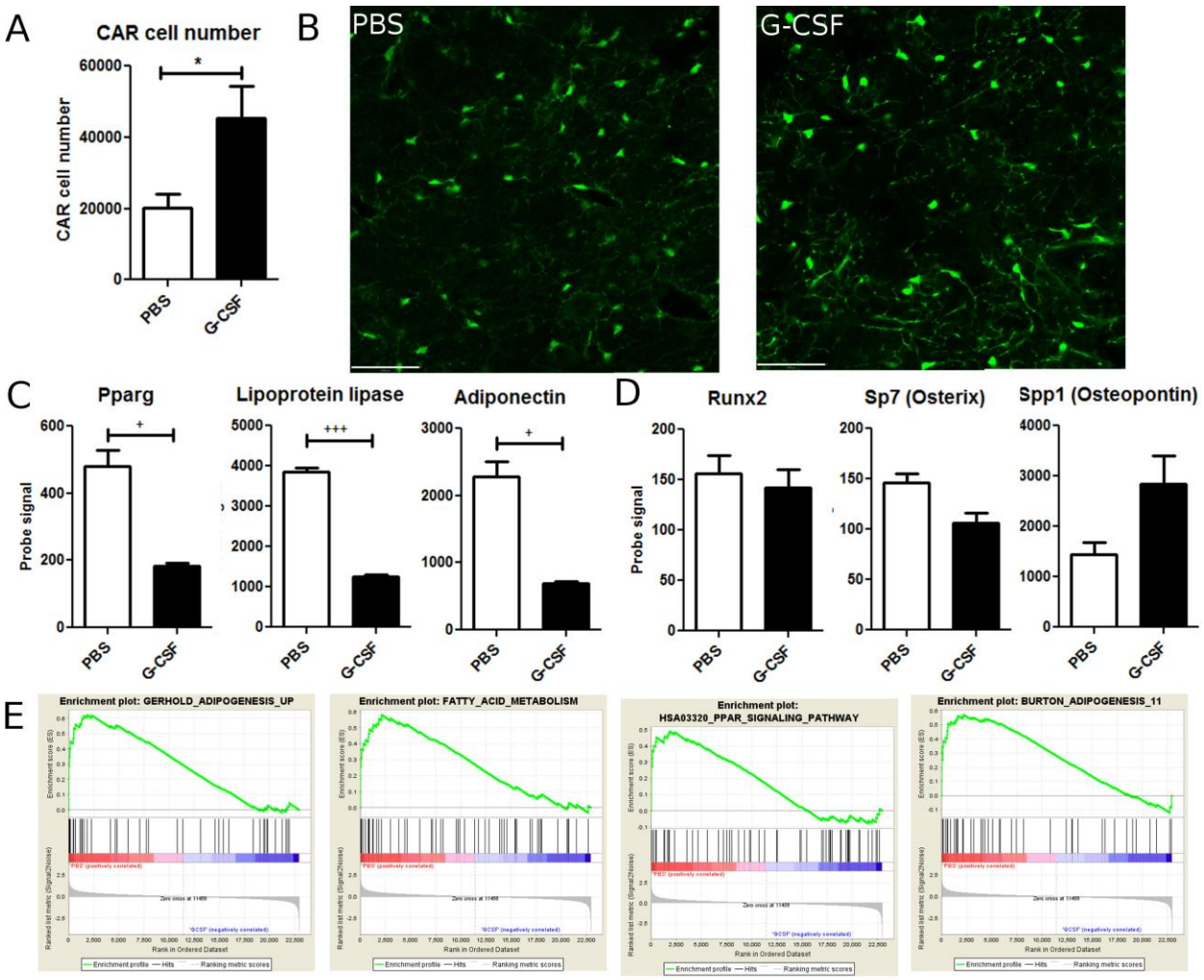

Figure 2.8. G-CSF reprograms B cell-supportive CAR cells. **A.** *Cxcl12*^{gfp} mice were treated for 7 days with G-CSF or PBS. **A.** The number of GFP^{hi} CAR cells was quantified by flow cytometry. **B**. Representative photomicrographs for PBS-treated (left panel) or G-CSF-treated (right panel) mice are shown. CAR cells express high levels of GFP (green). Scale bar: 100 µm. **C.** Probe signals from RNA expression profiling of sorted CAR cells are shown for the indicated adipogenic genes. **D.** Probe signals from RNA expression profiling of sorted CAR cells are shown for the indicated osteogenic genes. **E.** Gene Set Enrichment Analysis enrichment plots of adipogenic gene sets. Data represent the mean ± SEM of 4-11 mice. *P < 0.05; **P < 0.01; ***P < 0.001; ns: non-significant; +FDR < 0.05; +++FDR < 0.001.

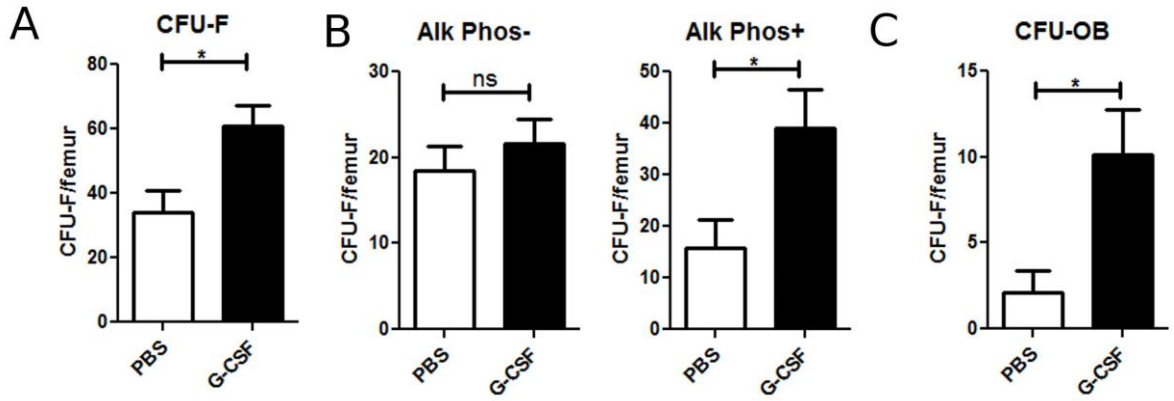

Figure 2.9. G-***CSF treatment increases bone marrow osteoblastic potential***. Wild type mice were treated for 7 days with PBS or G-CSF and bone marrow was plated into CFU-F culture. **A.** Total CFU-F activity at two weeks. **B.** The number of CFU-F cultures staining positive or negative for Alkaline Phosphatase is shown. **C.** The number of Von Kossa+ CFU-OB colonies at 3 weeks. Data represent the mean ± SEM of 7 mice. *P < 0.05; **P < 0.01; ***P < 0.001; ns: non-significant.

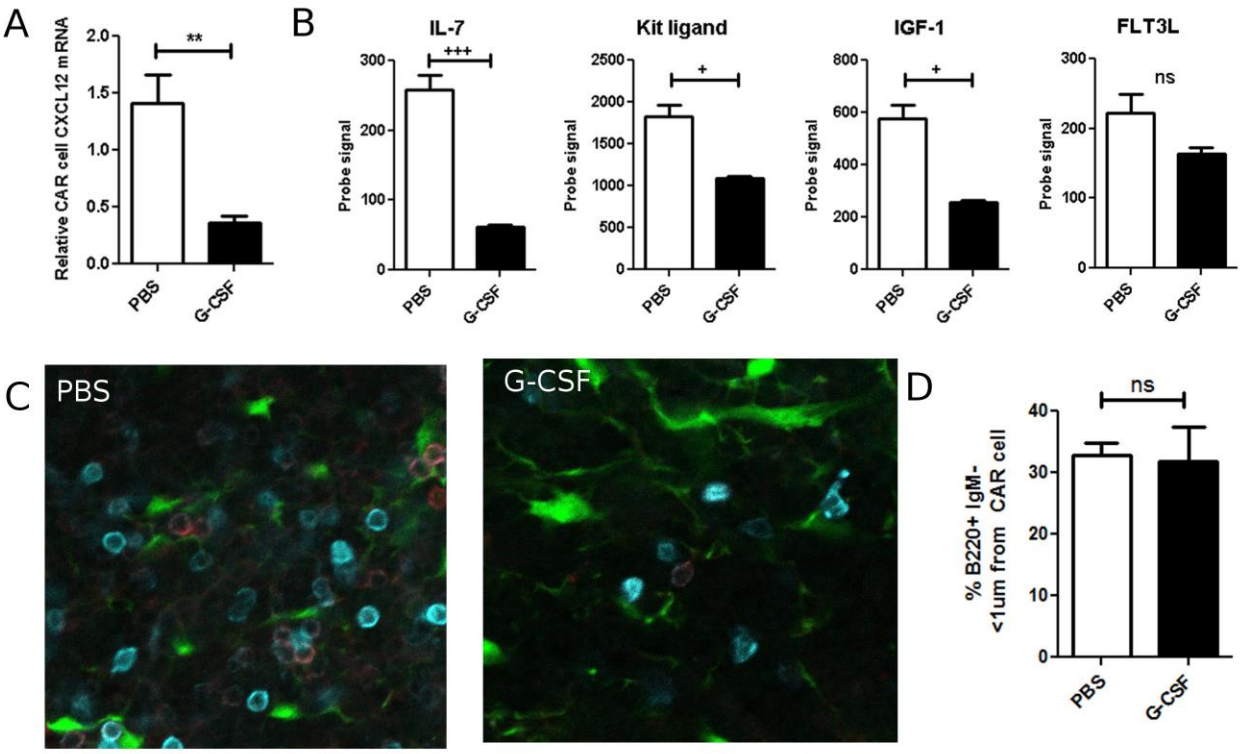

Figure 2.10. G-CSF targets expression of B trophic factors in CAR cells and osteoblasts. **A.** The expression of CXCL12 mRNA relative to β-actin mRNA in sorted CAR cells was determined **B.** Probe signals from RNA expression profiling of sorted CAR cells are shown for the indicated B cell trophic genes. **C.** Representative photomicrographs showing the relationship of CAR cells to B220+ (cyan) IgM- (red) B cell precursors. **D.** Quantification of the number of B220+ IgM- cells within 1 um of a CAR cell. Data represent the mean ± SEM of 3-5 mice. *P < 0.05; **P < 0.01; ***P < 0.001; ns: non-significant.

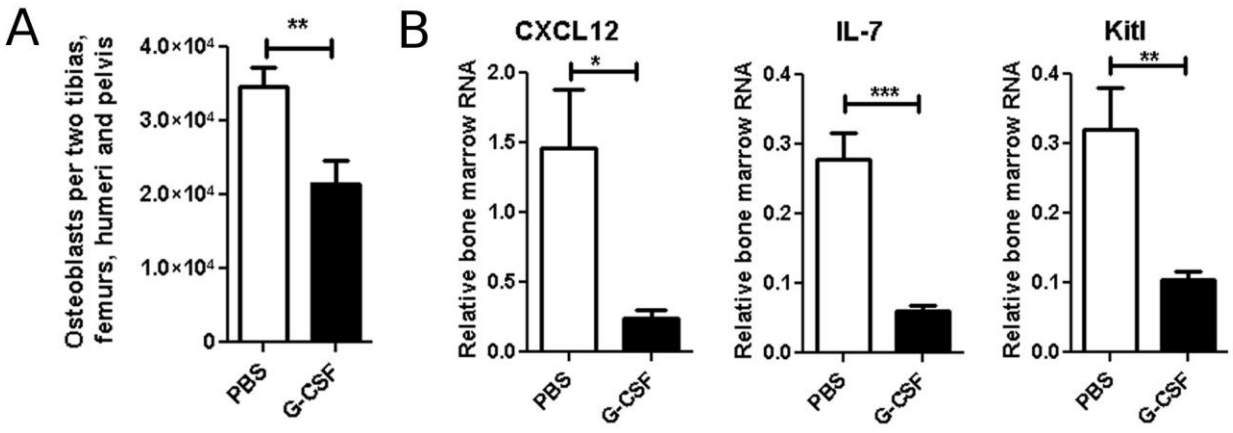

Figure 2.11. G-CSF suppresses osteoblast expression of B trophic factors. **A.** *Col2.3-Gfp* mice were treated for 7 days with G-CSF or PBS. The number of GFP^+ osteoblasts was enumerated by flow cytometry. **B.** The expression of the indicated genes relative to β-actin in mRNA from sorted osteoblasts is shown. Data represent the mean ± SEM of 7 mice. *P < 0.05; **P < 0.01; ***P < 0.001; ns: non-significant.

2.8. TABLES

Gene symbol	PBS	G-CSF
CXCL12	5248 ± 198.8	4081 ± 87.73
KITL	1826 ± 141.5	1087 ± 30.64
IL7	258 ± 20.46	61.14 ± 4.124
FLT3L	221.6 ± 28.32	164.1 ± 8.008
PPARγ	481.1 ± 46.10	182.1 ± 9.967
Lipoprotein Lipase	3861 ± 87,75	1260 ± 35.85
Adiponectin	2286 ± 217.8	699.5 ± 22.80
Runx2	156.0 ± 18.45	142.0 ± 17.82
Osterix	146.0 ± 9.599	106.5 ± 9.193
Osteopontin	1439 ± 249.4	2852 ± 556.9
Galectin-1	331.4 ± 95.51	594.9 ± 94.15
FOXC1	749.5 ± 74.61	429.1 ± 78.48

Table 2.1. CAR genechip expression profile. CAR cells were defined as GFP^{high} CD45- Ter119- CD31- cells from *Cxcl12-Gfp* bone marrow (n=4-5, mean ± SEM is shown).

CHAPTER 3: CXCL12 FROM BONE MARROW STROMAL CELLS REGULATES B CELL DEVELOPMENT IN A STAGE-SPECFIC MANNER

3.1. INTRODUCTION

CXCL12, through interaction with its major receptor CXCR4, plays an essential role in B cell development. Mice deficient for CXCL12 show an early defect in B lymphopoiesis and loss of developing B cells in the bone marrow[13]. The CXCL12-CXCR4 signaling axis also plays a role in human B cell diseases. CXCL12 signaling is important in engraftment and chemoresistance in B-ALL, while activating mutations in CXCR4 promote lymphoplasmacytic lymphoma proliferation, dissemination, and chemoresistance[31,119–122]. In the bone marrow, CXCL12 is constitutively expressed by several bone marrow stromal cell populations, including mesenchymal stem/progenitor cells, CAR cells, mature osteoblasts, and endothelial cells[63,66,70,73,74,79,131]. While bone marrow-derived CXCL12 is known to be important for B lymphopoiesis, it is not clear how CXCL12 derived from different stromal cell populations regulates B cell development. Dissecting how stromal-derived CXCL12 contributes to B lymphopoiesis will lead to a better understanding of how the stromal microenvironment regulates hematopoiesis and may provide insight into the complex relationships between different stromal cell populations. Since CXCL12-CXCR4 signaling is known to play a role in the maintenance of B cell malignancies, understanding which bone marrow stromal cells provide CXCL12 critical for specific stages of B lymphopoiesis could provide therapeutically useful insights.

3.2. MATERIALS AND METHODS

Mice. All mice were backcrossed at least 10 generations and maintained on a C57BL/6 background with the exception of *Cxcl12-Gfp* and *Dmp1-Cre* mice. Generation of *Cxcl12*^{flox}

mice has been previously described[70]. *Prx1-Cre*[132], *Osx-Cre*[133], *Tie-2-Cre*[134], CAG-GFP, and Ai9 reporter mice were obtained from The Jackson Laboratory. *OC-Cre* mice were a gift from Thomas Clemens (Johns Hopkins University, Maryland), *Cxcl12-Gfp* mice were a gift from Takashi Nagasawa (Kyoto University, Japan), *Cxcr4*^{flox} mice were a gift from Robin Klein (Washington University in St. Louis), and *Dmp1-Cre* mice were a gift from Roberto Civatelli (Washington University in St. Louis). *Cxcl12*$^{+/-}$ mice were obtained through the RIKEN BioResource Center (Ibaraki, Japan). Mice were maintained under standard pathogen free conditions according to methods approved by the Washington University Animal Studies Committee.

Bone marrow and spleen analysis. Bone marrow and spleen cells were harvested using standard techniques and quantified using a Hemavet automated cell counter (CDC Technologies) or Cellometer automated cell counter (Nexelcom).

Quantitative RT-PCR. For total bone marrow RNA, femurs or tibias were flushed with 1 mL of Trizol (Invitrogen) and RNA was prepared according to manufacturer's instructions. cDNA was prepared using SuperScript III (Invitrogen) or Taqman RT Enzyme (Applied Biosystems). qRT-PCR was performed using the TaqMan Universal RT Master Mix (Applied Biosystems) using no template and no RT controls. Data was collected on a 7300 Real-Time PCR System (Applied Biosystems). Primers were: CXCL12 forward, 5'-GAGCCAACGTCAAGCATCTG-3'; CXCL12 reverse, 5'-CGGGTCAATGCACACTTGTC-3'; CXCL12 dT-FAM/TAMRA probe, 5'-TCCAAACTGTGCCCTTCAGATTGTTGC-3'; β-actin forward, 5'-

ACCAACTGGGACGATATGGAGAAGA-3'; β-actin primer; β-actin dT-VIC/TAMRA probe, 5′-AGCCATGTACGTAGCCATCCAGGCTG-3′.

Flow cytometry. Flow cytometry data were collected on Gallios 10-color, 3-laser or 4-laser flow cytometers (Beckman Coulter) and analyzed with FlowJo software (Treestar). Cells were stained by standard protocols with the following antibodies (eBiosciences unless otherwise noted): CD3e (145-2C11), CD45R (RA3-6B2, B220), CD11c (N418), Flk2 (A2F10), CD27 (LG.7F9), IL-7Ra (gift of Deepta Bhattacharya, Washington University), Ly6D (49-H4, BD Biosciences), NK1.1 (PK136), IgM (II/4), IgD (11-26c), CD19 (eBio1D3), CD43 (S7, BD Biosciences), TER-119, CD45 (30-F11), and CD31 (390).

Stromal cell analysis. To extract bone marrow stromal cells, femurs and tibias were crushed with a mortar and pestle in phosphate buffered saline (PBS). Cells in suspension were collected and stored on ice while bone chips and aggregates were digested using collagenase type II (3mg/mL, Worthington Biochemical) and dispase (4mg/mL, Roche) at 37°C for 1 hour in a shaking water bath. Following digestion both fractions were pooled, red blood cells lysed, and analyzed. CAR cells were defined as $CD45^{-}$ $TER119^{-}$ $CD31^{-}$ GFP^{bright} cells in mice carrying the *Cxcl12*^{gfp} transgene. Cre-targeted cells were defined as $TdTomato^{+}$ cells in mice carrying the *lox-stop-lox-TdTomato Ai9* transgene.

Immunostaining of bone sections. Femurs and tibias were harvested and fixed for 16-24 hours in 4% paraformaldehyde (Sigma-Aldrich) at 4°C. Bones were washed twice in PBS, decalcified in 14% ethylenediaminetetraacetic acid (EDTA) pH 7.4 solution for 3-5 days, and cryoprotected

in 30% sucrose in PBS for 16-24 hours. Bones were then snap frozen in OCT media (Tissue-Tek), and tissue blocks were sectioned (10 µm or 100 µm) using the CryoJane (Leica Biosystems). Antibodies used included: goat anti-mouse VE-Cadherin (R&D Systems AF1002), rat anti-mouse IgD (BD Biosciences clone 11-26c.2a), rat anti-mouse B220 (BD-Biosciences RA3-6B2), rat anti-mouse IgM (eBioscience II/4), and mouse anti-alpha smooth muscle actin (Sigma Aldrich 1A4). Sections were washed in TNT buffer (0.1 M Tris-HCl, 0.15 M NaCl, 0.05% Tween-20) and mounted with Prolong Gold Antifade Reagent (Invitrogen). Slides were imaged using a LSM 700 confocal microscope and ZEN imaging software (Zeiss).

Mature B cell homing assay. Spleens were harvested from 8-12 week old CAG-GFP mice and dissociated into a single cell suspension. 5 million splenocytes were injected retro-orbitally into *Cxcl12*$^{flox/-}$ or *OC-Cre Cxcl12*$^{flox/-}$ mice. Recipients were harvested 24 hours after transplantation, and GFP^{+} mature naïve B cell number was quantified in the bone marrow and spleen via flow cytometry.

Statistics. Significance was determined using Prism software (GraphPad). Unless otherwise stated, statistical significance of differences was calculated for two groups using Students t-test and 3 or more groups using 1- or 2-way ANOVA. P-values less than 0.05 were considered significant. All data are presented as mean ± SEM.

3.3. RESULTS

3.3.1. Specific stromal cell populations regulate distinct stages of B cell development.

To determine the role CXCL12 from different bone marrow stromal cells plays in B lymphopoiesis, we used a transgenic mouse line generated in our lab carrying a floxed *Cxcl12* allele, allowing conditional deletion of *Cxcl12* in specific stromal cell populations. *Cxcl12*^{flox} mice were crossed with mice expressing different Cre recombinases to target different stromal cell populations. Deletion of *Cxcl12* within endothelial cells using *Tie2-Cre*[134] did not affect baseline bone marrow B lymphopoiesis. *Prx1-Cre* was used to conditionally delete *Cxcl12* in mesenchymal stem cells and their downstream progeny[70,132], including CAR cells, osteoblasts, and osteocytes. Deletion of *Cxcl12* in *Prx1-Cre*-targeted cells almost completely ablated bone marrow CXCL12 mRNA production and severely reduced bone marrow white blood cell count and B cell number (Figure 3.1A, 3.1B, & 3.1C). All stages of bone marrow B cell development were reduced, beginning at the common lymphoid progenitor (CLP) stage of lymphopoiesis (Figure 3.1C & 3.1D). *Osterix-Cre* (*Osx-Cre*) was used to delete *Cxcl12* in CAR cells and their downstream progeny[70,133], resulting in a severe decrease in bone marrow CXCL12 mRNA expression and bone marrow cellularity. *Osx-Cre Cxcl12*$^{flox/-}$ mice have normal numbers of CLPs and B-committed lymphoid progenitor (BLPs) but have reduced number of bone marrow B cells beginning at the pre-pro-B stage of B lymphopoiesis. *Osteocalcin-Cre* (*OC-Cre*) was used to delete *Cxcl12* in osteolineage cells[86]. Deletion of *Cxcl12* using *OC-Cre* did not significantly alter overall bone marrow CXCL12 expression, suggesting that osteolineage cells do not contribute significantly to bulk CXCL12 production in the bone marrow. However, despite the relatively minor contribution of osteolineage cells to overall CXCL12 production in the bone marrow, deletion of *Cxcl12* using *OC-Cre* resulted in a significant loss of mature naïve B cells in the bone

marrow. These data suggest that CXCL12 supports B cell development in a stage- and stromal-cell specific fashion. CXCL12 production from MSCs is important for CLPs, CXCL12 from CAR cells is required for the generation of early B lymphoid cells, CXCL12 production from osteolineage cells is required to maintain mature naïve B cell number in the bone marrow, and CXCL12 from endothelial cells is dispensable for baseline B cell development.

3.3.2. *OC-Cre*-deletion of *Cxcl12* does not affect bone marrow dendritic cell number

Perivascular dendritic cells have been reported to be a niche cell supporting mature naïve B cell number in the bone marrow. Ablation of dendritic cells results in decreased mature B cell survival in the bone marrow but does not affect homing[93]. To determine if loss of dendritic cells is responsible for the observed decrease in bone marrow mature naïve B cell, we enumerated $CD11c^+$ dendritic cells in the bone marrow of *OC-Cre* $Cxcl12^{flox/-}$ mice relative to control mice. We detected no difference in bone marrow dendritic cell number (Figure 3.2), indicating that deletion of *Cxcl12* in *OC-Cre*-targeted cells does not result in decreased dendritic cells.

3.3.3. CXCL12 expression in *OC-Cre*-targeted cells is required for efficient homing of mature B cells to the bone marrow.

Mature naïve B cells home to the bone marrow after undergoing maturation in peripheral lymphoid organs. The number of mature B cells in the spleen of *OC-Cre* $Cxcl12^{flox/-}$ mice is comparable to control mice (Figure 3.3A), suggesting that the loss of mature B cells in the bone marrow of *OC-Cre* $Cxcl12^{flox/-}$ mice is not due to defective peripheral maturation. We examined the localization of mature B cells in the bone marrow of wild type and *OC-Cre* $Cxcl12^{flox/-}$ mice to determine if mature B cells had an altered localization due to loss of CXCL12 derived from

OC-Cre-targeted cells. As previously reported, mature B cells are predominantly localized to perivascular niches in the bone marrow, and we detected no major mature B population in direct contact with osteoblasts. Though markedly reduced in number, mature B cells in *OC-Cre Cxcl12*$^{flox/-}$ animals exhibit similar perivascular localization (Figure 3.3B). We next examined the homing of mature B cells to the bone marrow. Splenocytes from CAG-GFP mice, which ubiquitously express GFP, were adoptively transferred into *Cxcl12*$^{flox/-}$ and *OC-Cre Cxcl12*$^{flox/-}$ mice, and the number of GFP^{+} mature B cells in the bone marrow and spleen was assessed 24 hours later (Figure 3.3C). Homing of mature B cells to the bone marrow, but not to the spleen, of *OC-Cre Cxcl12*$^{flox/-}$ mice was significantly impaired compared with control mice (Figure 3.3D). Interestingly, of the mature B cells that did home to the bone marrow, their localization to perivascular regions was similar in *Cxcl12*$^{flox/-}$ compared to *OC-Cre Cxcl12*$^{flox/-}$ recipient mice (Figure 3.3E). Together, these data show that CXCL12 expression from *OC-Cre*-targeted cells plays a key role in the homing and/or retention of mature B cells in the bone marrow.

3.3.4. Autocrine CXCL12-CXCR4 signaling in *OC-Cre*-targeted cells is not required for normal B lymphopoiesis.

CXCR4 signaling is required for normal osteoblast function[135], and deletion of *Cxcr4* within osteolineage cells has been shown to affect hematopoiesis. To test the hypothesis that loss of autocrine CXCL12-CXCR4 signaling in the *OC-Cre*-targeted compartment is responsible for decreased mature naïve B cell homing, we generated *OC-Cre Cxcr4*$^{flox/flox}$ mice to conditionally delete *Cxcr4* from cells expressing *OC-Cre*. We detected no alteration in mature naïve B cell number in the bone marrow (Figure 3.4), indicating that autocrine CXCL12-CXCR4 is not essential for mature naïve B cell homing to the bone marrow.

3.3.5. *OC-Cre* targets osteocytes, osteoblasts, central marrow cells, and perivascular arteriolar smooth muscle cells

Osteocalcin expression is highest in mineralizing osteoblasts but *OC-Cre* has been reported to target central marrow stromal cells as well[86]. To better describe the cell populations targeted by *OC-Cre*, we performed lineage mapping studies using Ai9 fluorescent reporter mice. Recombination was observed in osteocytes, endosteal osteoblasts, scattered central marrow cells, and periarteriolar alpha smooth actin-positive pericytes (Figure 3.5A & 3.5B). To determine if *OC-Cre*-targeted central marrow cells overlapped with CAR cells, we generated triple-transgenic *OC-Cre Cxcl12-Gfp Ai9* mice. While there is substantial overlap between *OC-Cre*-targeted cells and CAR cells (68.8 ± 4.7% of CAR cells are TdTomato^{+}), a significant minority of CAR cells are not targeted by *OC-Cre* (30.9 ± 5.2%) (Figure 3.6A, 3.6B, & 3.6C). Similarly, most Ai9 cells are CAR cells (59.3 ± 8.3%), but a significant percentage of Ai9^{+} cells are GFP^{-} (30.6 ± 8.9%) or GFP^{intermediate} (9.8 ± 0.1%) (Figure 3.6A, 3.6B, and & 3.6D), indicating that *OC-Cre* targets some, but not all, CAR cells as well as a population of GFP intermediate stromal cells.

3.3.6. CXCL12 production from osteoblasts and osteocytes is not required for normal B lymphopoiesis

Osteoblasts and osteocytes are known to play an important role in B lymphopoiesis[76,81–83]. To test if CXCL12 from osteoblasts and osteocytes is responsible for the B cell phenotype observed in *OC-Cre Cxcl12$^{flox/-}$* mice, we used *Dmp1-Cre* to delete *Cxcl12* in mature osteoblast populations. While *Dmp1-Cre* was originally described to target osteocytes embedded in bone matrix[136], recent reports suggest that *Dmp1-Cre* also targets endosteal osteoblasts[137,138]. To determine which cell types are targeted by *Dmp1-Cre*, we performed lineage mapping studies.

As previously reported, *Dmp1-Cre* efficiently recombined in osteocytes and endosteal osteoblasts. Surprisingly, we also found that *Dmp1-Cre* targeted scattered central marrow cells (Figure 3.7A), indicating that *Dmp1-Cre* targets more broadly than has previously been reported. In contrast to *OC-Cre*, however, perivascular smooth muscle cells were very rarely targeted. The number of mature naïve B cells in the bone marrow was unaffected in *Dmp1-Cre Cxcl12*$^{flox/-}$ mice (Figure 3.7B), indicating that CXCL12 production from endosteal osteoblasts and osteocytes is not essential for mature naïve B cell homing.

3.3.7. G-CSF suppresses B lymphopoiesis, in part, in a CXCL12 independent fashion

In Chapter 2, we show that G-CSF decreases B cell number in the bone marrow. While decreased CXCL12 production by stromal cells clearly contributes to G-CSF-induced B cell suppression, it is unclear whether the loss of B cells is entirely attributable to decreased CXCL12 expression. To address this issue, we analyzed the effect of G-CSF treatment on *Prx1-Cre Cxcl12*$^{flox/-}$ or control mice. Of note, CXCL12 mRNA production in *Prx1-Cre Cxcl12*$^{flox/-}$ mice is nearly undetectable, thus any change in B cell number induced by G-CSF likely would be CXCL12 independent. In control mice, G-CSF treatment resulted in a significant decrease in bone marrow B cells. As noted already, the basal number of B cells in the bone marrow of *Prx1-Cre Cxcl12*$^{flox/-}$ mice was significantly reduced. However, B cell number was further reduced with G-CSF treatment in these mice (Figure 3.8). Thus, G-CSF treatment acts in both a CXCL12-dependent and CXCL12-independent fashion to suppress B lymphopoiesis.

3.4. DISCUSSION

Previous studies have demonstrated the importance of CXCL12 in B lymphopoiesis.

Mice deficient for CXCL12 or its primary receptor, CXCR4, have decreased B cell number in the bone marrow beginning prior to pre-pro-B stage, due in part to reduced retention of developing B cells in the bone marrow[13–15,19,203–16]. Chimeric mice and conditional deletion of *Cxcr4* show that CXCL12 from the stromal compartment is required for normal B lymphopoiesis and for mature B cell localization to the bone marrow[18]. Within the bone marrow, CXCL12 is produced by multiple different stromal cell populations, including mesenchymal stem cells, CAR cells, osteoblasts, and endothelial cells[63,66,70,73,74,79,131], and some of these populations are known to play important roles in B lymphopoiesis. Pre-pro-B cells have been reported to localize to CXCL12-expressing CAR cells in bone marrow[63], and ablation of CAR cells results in decreased CLP number, decreased pro-B proliferation, and increased pro-B apoptosis[74]. Disruption of the transcription factor *Foxc1* disrupts CAR cell osteogenic potential, downregulates CXCL12 production, and inhibits B lymphopoiesis[75]. Osteoblasts can support B cell proliferation and differentiation *in vitro*, and ablation of osteoblasts or conditional deletion of signaling molecules within osteoblasts can disrupt B lymphopoiesis[76,81,82]. Osteocytes have also been implicated in the regulation of bone marrow B cell development via production of sclerostin, important for B cell survival[83]. The specific role of CXCL12 within these populations on B lymphopoiesis, however, has not previously been reported.

We show that CXCL12 expression from mesenchymal progenitors is required for maintenance of CLP number, while CXCL12 from CAR cells is not required until the pre-pro-B cell stage of B cell differentiation. Our data also show that CXCL12 expression from *OC-Cre*-targeted cells is required to maintain the reservoir of mature naïve B cells in the bone marrow via homing from the peripheral circulation and retention in the bone marrow. These results are somewhat surprising, since CXCL12 production from osteoblasts/osteocytes is a minor

contributor to bulk CXCL12 production in the bone marrow. Because dendritic cells are known to be important for mature B cell survival in the bone marrow[93], we examined dendritic cell number in *OC-Cre Cxcl12*$^{flox/-}$ mice and detected no change in dendritic cell number. A recent study showed that deletion of *Cxcr4* in mature osteoblasts resulted in impaired osteoblast function[135], suggesting that CXCL12 production by osteoblasts/osteocytes may act in an autocrine fashion to regulate function. Autocrine CXCL12-CXCR4 signaling within the *OC-Cre*-targeted compartment in not required for mature naïve B cell homing, however, since no defect is observed in *OC-Cre Cxcr4*$^{flox/flox}$ mice. Further research is required to understand how CXCL12 from *OC-Cre*-targeted cells results in decreased mature B cell homing and retention, whether through a direct mechanism of CXCL12 from the *OC-Cre*-compartment interacting with CXCR4 on mature recirculating B cells, or via an indirect mechanism.

Although prior studies suggested that *OC-Cre* specifically targeted mature osteoblasts and osteocytes[86], our lineage-mapping studies show that *OC-Cre* targets osteocytes, osteoblasts, central marrow cells, and periarteriolar smooth muscle cells. Deletion of *Cxcl12* within osteoblasts and osteocytes using *Dmp1-Cre* did not affect mature naïve B cell number in the bone marrow, indicating that CXCL12 from these populations is not required for mature naïve B cell homing. *Dmp1-Cre* does not target periarteriolar smooth muscle cells, however, a compartment that is targeted by *OC-Cre*. Periarteriolar smooth muscle cells have not previously been described to play a role in B lymphopoiesis, but recent evidence has emerged suggesting they may play a role in stem cell maintenance. It has also been reported that they express high levels of CXCL12 and KITL[85], known to be important for bone marrow B cell development, suggesting that they may play a role in bone marrow B lymphopoiesis, and loss of CXCL12 within this population may be sufficient to result in impaired mature B cell homing to the bone

marrow. Central marrow cells targeted by *OC-Cre* could also be responsible for the homing defect observed in *OC-Cre Cxcl12*$^{flox/-}$ mice. While both *OC-Cre* and *Dmp1-Cre* target a reticular central marrow stromal cell population, it is currently unknown if the cell type, cell number, and extent of targeting is similar in the two models. Further study will be needed to rule out the possibility that CXCL12 produced by these central marrow cells is responsible for mature naïve B cell homing.

A study on an independent *Cxcl12* conditional deletion mouse model provides complementary insight on the role of CXCL12 from different stromal cell populations on B lymphopoiesis. In that study, *Col2.3-Cre* was used to delete *Cxcl12* in osteoblasts. Lineage mapping data was not reported, so it is unknown to what extent *Col2.3-Cre* overlaps with *OC-Cre* or *Dmp1-Cre*. Under baseline conditions, CLP number was decreased but downstream committed B cells, including pre-pro-B, pro-B, and pre-B cells, were normal in number. Mature naïve B cell number was not enumerated. Lin$^-$ IL-7Ra$^+$ cells, a population enriched for CLP and lymphoid-primed multipotent progenitors, were reported by immunofluorescence to be enriched at the endosteum, suggesting that endosteal osteoblasts may play a special role in maintaining CLP, and that in spite of low CLP number, CXCL12 from other niches is sufficient to normalize later stages of B cell development. Upon transplant into irradiated wild type recipients, *Col2.3-Cre Cxcl12*$^{flox/flox}$ bone marrow had significantly reduced contribution to B cell lineage relative to control cells, suggesting reduced B cell potential as late as 16 weeks post-transplant[73]. It is unclear why a deficit in B lymphopoiesis would be maintained after transplant into an environment expressing wild type levels of CXCL12 when B cell number is normal *Col2.3-Cre Cxcl12*$^{flox/flox}$ mice. It is possible that stress induced by transplant is sufficient to overwhelm the compensatory mechanisms that are sufficient to normalize B cell number under non-transplant

conditions. In the same study, *Lepr-Cre* was used to delete *Cxcl12* in CAR cells. In contrast to *Osterix-Cre*, no loss of bone marrow B cells was observed. The discrepancy may be due to wider targeting in *Osterix-Cre* mice, where osteoblasts, osteocytes, and periarteriolar smooth muscle cells are targeted, whereas these populations were not targeted in *Lepr-Cre* mice at the time the mice were analyzed, or different targeting efficiency within the CAR cell compartment.

Our data are consistent with a model of B lymphopoiesis in which developing B cells in the bone marrow interact with different stromal cell niche populations as the B cells differentiate. Data supporting this model comes from multiple different groups reporting that bone marrow B cells at different stages of differentiation are in contact with different stromal cell populations, including data suggesting that CLPs are in contact with endosteal osteoblasts[73], pre-pro-B cells are in contact with CAR cells, pro-B cells are in contact with IL-7-expressing cells[63], late pre-B cells are in contact with Galectin-1-expressing cells[64], and mature naïve B cells are in contact with dendritic cells[93]. In this model, our data would suggest that CXCL12 provided by MSCs is essential for CLP maintenance, CXCL12 from CAR cells is important for pre-pro-B cell development, and CXCL12 from an *OC-Cre*-compartment is required for mature naïve B cell homing. Because bulk CXCL12 levels are different in each of our mouse models, however, our data are also consistent with an alternative model in which different B cell populations are differentially sensitive to overall bone marrow CXCL12 level. In this model, mature naïve B cell homing are sensitive to modest reductions in overall bone marrow CXCL12 level, pre-pro-B cells are sensitive to an intermediate reduction in overall bone marrow CXCL12 level, and CLPs are sensitive only to a severe reduction in total bone marrow CXCL12. A rescue experiment in which overall CXCL12 level in the bone marrow is restored from a non-physiologic source or

where CXCL12 expression is restored in some but not all niche populations could distinguish between these models.

Stromal cells in the bone marrow are currently poorly understood, and dissecting the identity of the cells differentially targeted, and their relationship to one another, has the potential to clarify the nature of the bone marrow microenvironment. Lineage mapping of *OC-Cre Ai9 Cxcl12-Gfp* mice showed that the *OC-Cre*-targets multiple stromal cell populations, including $GFP^{intermediate}$ and GFP^{-} cell populations. Comparison with similar populations in *Dmp1-Cre Ai9 Cxcl12-Gfp* mice or surface phenotyping may provide clues as to the identity of these cells. The targeted compartment in *OC-Cre* mice also included some, but not all, CAR cells. The biological relevance of these results is not currently known. Since CAR cells are a multipotent osteogenic-adipogenic population, it is possible that CAR cells targeted by *OC-Cre* have committed to the osteoblastic lineage, representing an intermediate stage between CAR cells and osteoblasts. *OC-Cre*-targeted CAR cells could also represent a distinct lineage from non-targeted CAR cells, however. Periarteriolar smooth muscle cells have also been reported to be an osteoprogenitor population[84], and our lineage mapping data are consistent with that model. CAR cells and periarteriolar smooth muscle cells may represent two independent methods of generating osteoblasts, or one population could be descended from the other. Based on lineage mapping data, if CAR cells and periarteriolar cells are related, CAR cells are likely descended from periarteriolar cells rather than the converse, since *OC-Cre* targets periarteriolar cells and some central marrow CAR cells, while *Dmp1-Cre* efficiently targets central marrow cells but not periarteriolar cells. This model is also consistent with data indicating that *Lepr-Cre* targets CAR cells and osteoblasts but not periarteriolar smooth muscle cells[71].

These data show that CXCL12 derived from distinct bone marrow stromal cells has a differential effect on bone marrow B lymphopoiesis. Clarifying which stromal cell populations regulate B cell development has potential relevance to human disease. CXCL12 is known to be an important niche factor regulating B cell diseases corresponding to different stages of B cell development. CXCL12 is known to be an important bone marrow niche component for B-ALL, the most common subset of which corresponds to the pre-B stage of B lymphopoiesis, while activating CXCR4 mutations are common in lymphoplasmacytic lymphoma and IgM-MGUS, corresponding to later stages of B cell maturation[31,118–122]. Understanding which stromal cells provide CXCL12 crucial for the maintenance of these different populations opens the possibility of developing targeted therapy to disrupt interactions between malignant cells and their niches while limiting toxicity to non-malignant B cells and other bone marrow niches.

3.5 ACKNOWLEDGEMENTS

We thank Jill Woloszynek, Amy Schmidt, and Molly Romine for technical assistance, Jackie Tucker-Davis for animal care, William Eades, Jackie Hughes, and Dan Schweppe for cell sorting assistance, Dr. Thomas Clemens for the *OC-Cre* mice, Dr. Roberto Civatelli for the *Dmp1-Cre* mice, and Dr. Robin Klein for the *Cxcr4*^{flox} mice. Microscopy imaging support was provided by Angie Schrader and the Microscopy and Digital Imaging Core of the Research Center for Auditory and Vestibular Studies, NIH P30DC004665. This work was supported by NIH grants GM07200, RO1 HL60772 (DCL) and F30 HL112552-02 (RBD).

3.6 AUTHOR CONTRIBUTIONS

Ryan B. Day designed and performed the research, analyzed the data, and wrote the manuscript. Michael Zhang assisted in the analysis of *Dmp1-Cre Cxcl12*^{flox} and *OC-Cre Cxcr4*^{flox} mice. Adam Greenbaum generated the *Cxcl12*^{flox} mice and contributed some data to the CXCL12 RNA expression analysis. Deepta Bhattacharya provided technical assistance and assisted with data analysis. Takashi Nagasawa provided the *Cxcl12-Gfp* mice. Daniel C. Link supervised all of the research and edited the manuscript.

3.7. PUBLICATION

Part of this work was published in: Greenbaum A, Hsu YM, Day RB, Schuettpelz LG, Christopher MJ, Borgerding JN, Nagasawa T, Link DC. 2013. CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. Nature, 495(7440):227-30.

3.8 FIGURES

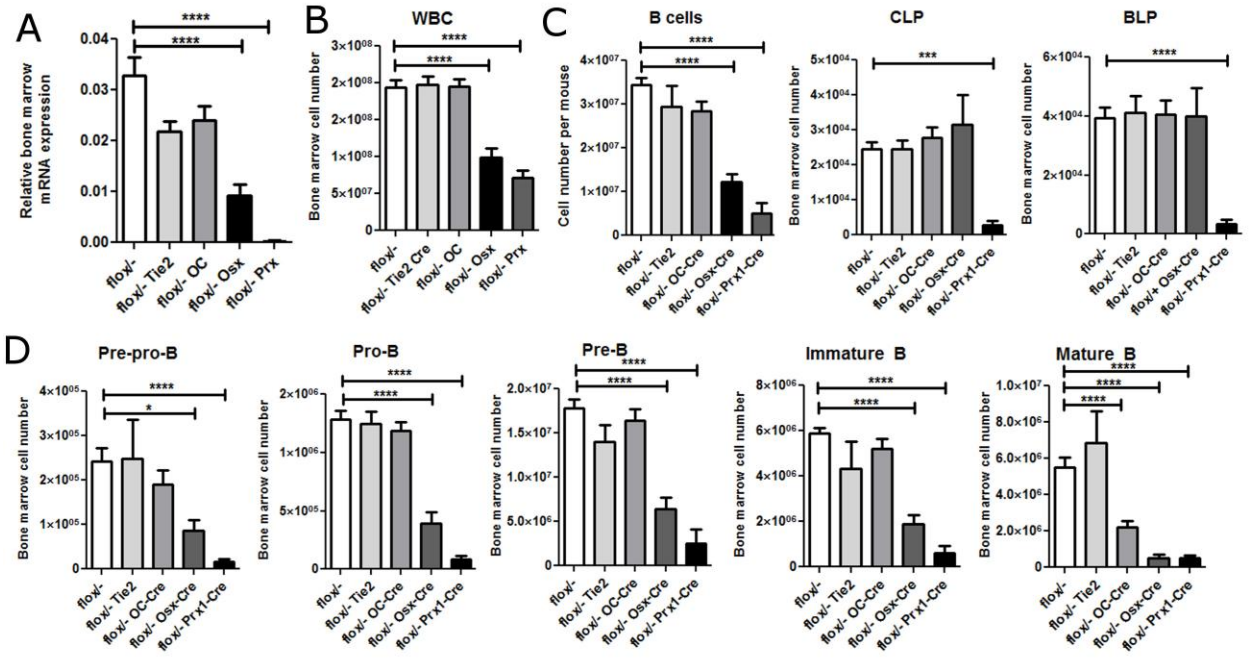

Figure 3.1. Loss of stromal cell production of CXCL12 results in impaired B lymphopoiesis.

A. Femurs from mice with the indicated genotypes were flushed with Trizol to extract total bone marrow RNA. Shown is the expression of CXCL12 mRNA relative to β-actin. **B.** The number of the indicated progenitor or B-cell subset in the bone marrow of *Cxcl12*$^{fl/-}$, *Tie2-Cre Cxcl12*$^{fl/-}$, *OC-Cre Cxcl12*$^{fl/-}$, *Osx-Cre Cxcl12*$^{fl/-}$, or *Prx1-Cre Cxcl12*$^{fl/-}$ mice was enumerated by flow cytometry **C & D.** The number of the indicated progenitor or B-cell subset in the bone marrow of *Cxcl12*$^{fl/-}$, *Tie2-Cre Cxcl12*$^{fl/-}$, *OC-Cre Cxcl12*$^{fl/-}$, *Osx-Cre Cxcl12*$^{fl/-}$, or *Prx1-Cre Cxcl12*$^{fl/-}$ mice was enumerated by flow cytometry. Data represent the mean ± SEM of 5-37 mice. *P < 0.05; **P < 0.01; ***P < 0.001; ns: non-significant.

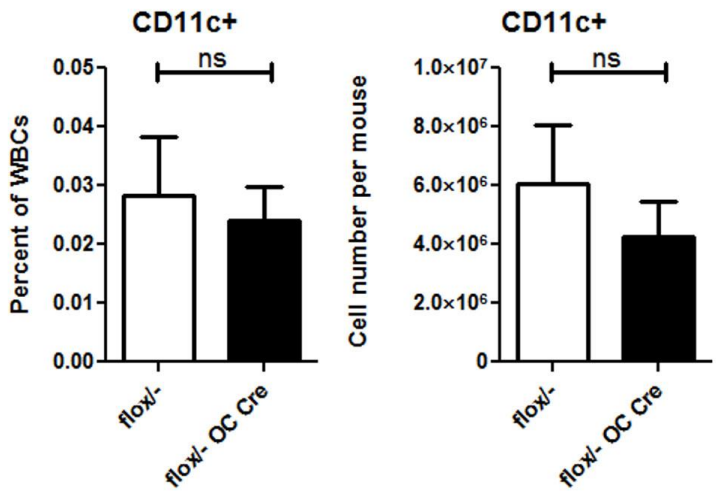

Figure 3.2. Cxcl12 deletion in OC-Cre-targeted cells does not affect dendritic cell number.

Shown is the bone marrow frequency (left panel) or absolute number (right panel) of CD11c+ dendritic cells in the bone marrow of Cxcl12$^{fl/-}$ or *OC-Cre Cxcl12*$^{fl/-}$ mice. Data represent the mean ± SEM of 7-15 mice. *P < 0.05; **P < 0.01; ***P < 0.001; ns: non-significant.

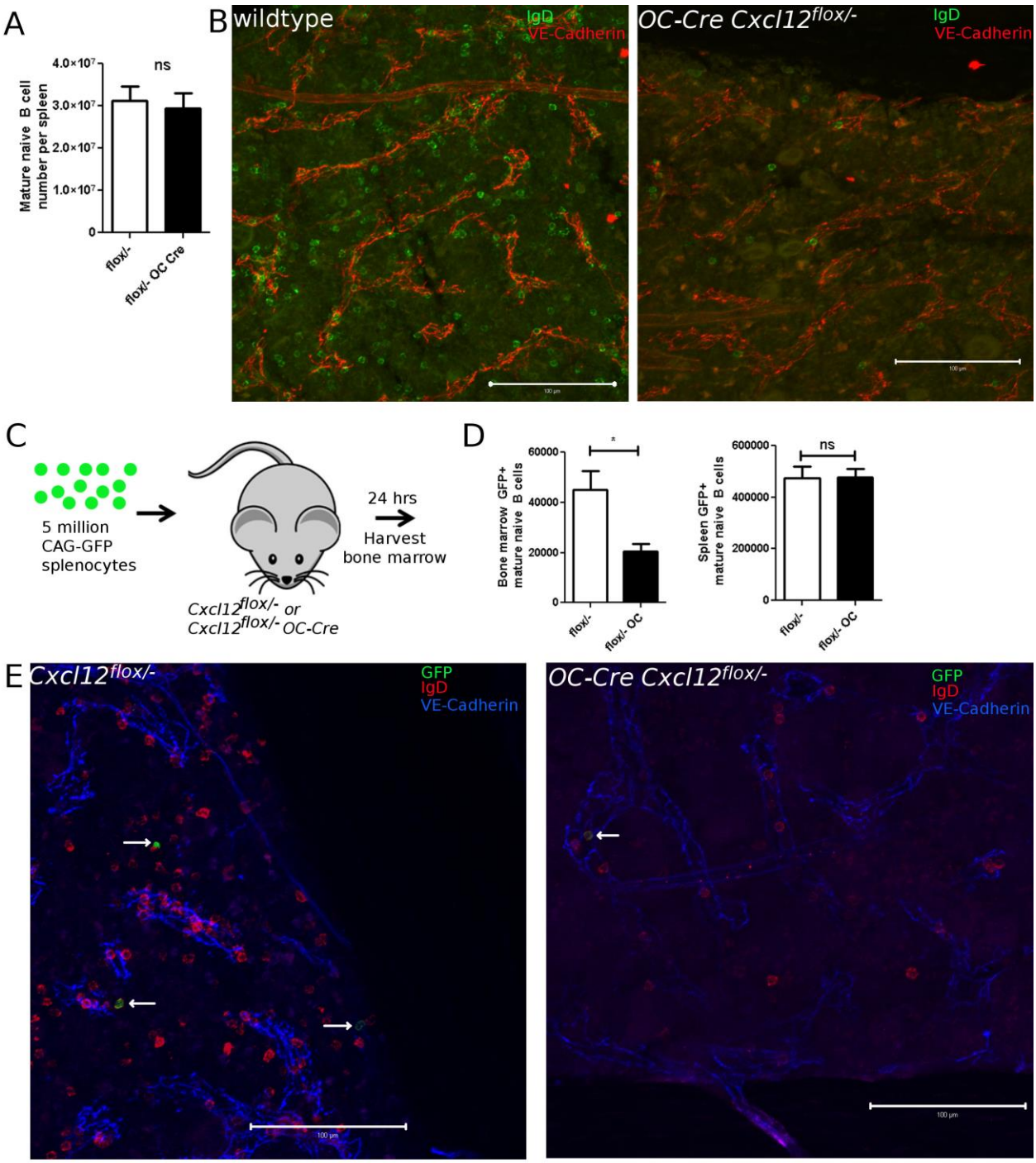

Figure 3.3. Homing of mature B cells to the bone marrow is impaired in OC-Cre Cxcl12$^{flox/-}$ mice. **A.** The number of mature (Fraction F) B cells in the spleen of mice of the indicated genotypes is shown. **B.** Representative photomicrographs showing the localization of mature B cells in the bone marrow of wild type (left panel) or *OC-Cre Cxcl12$^{flox/-}$* mice (right panel). IgD

expressing mature B cells (green) and VE-cadherin^{+} endothelial cells (red) are shown. Scale bar: 100 µm. **C.** Splenocytes (5 x 10^{6}) from CAG-GFP mice were injected intravenously into *OC-Cre Cxcl12$^{flox/-}$* or control mice. **D**. Shown is the number of GFP^{+} mature B cells in the bone marrow (left panel) and spleen (right panel) and 24 hours after injection. Data represent the mean ± SEM of 11-16 mice. *P < 0.05; ns: non-significant. **E.** Representative photomicrographs showing the localization of homed Fraction F cells in the bone marrow of *Cxcl12$^{flox/-}$* (left panel) or *OC-Cre Cxcl12$^{flox/-}$* mice (right panel). GFP expressing cells (green), IgD expressing mature B cells (red), and VE-cadherin^{+} endothelial cells (blue) are shown. Arrows indicate cells co-expressing GFP and IgD. Scale bar: 100 µm.

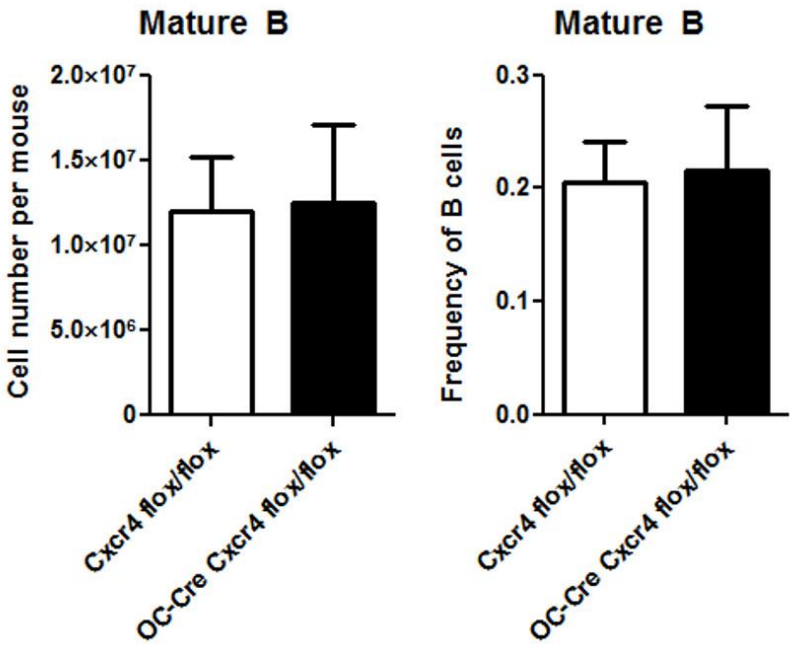

Figure 3.4. Autocrine CXCL12-CXCR4 signaling in OC-Cre-targeted cells is not required for normal B lymphopoiesis. Shown is the bone marrow frequency (left panel) or absolute number (right panel) of CD11c+ dendritic cells in the bone marrow of *Cxcl12*$^{fl/-}$ or *OC-Cre Cxcl12*$^{fl/-}$ mice. Data represent the mean ± SEM of 7-8 mice. *P < 0.05; **P < 0.01; ***P < 0.001; ns: non-significant.

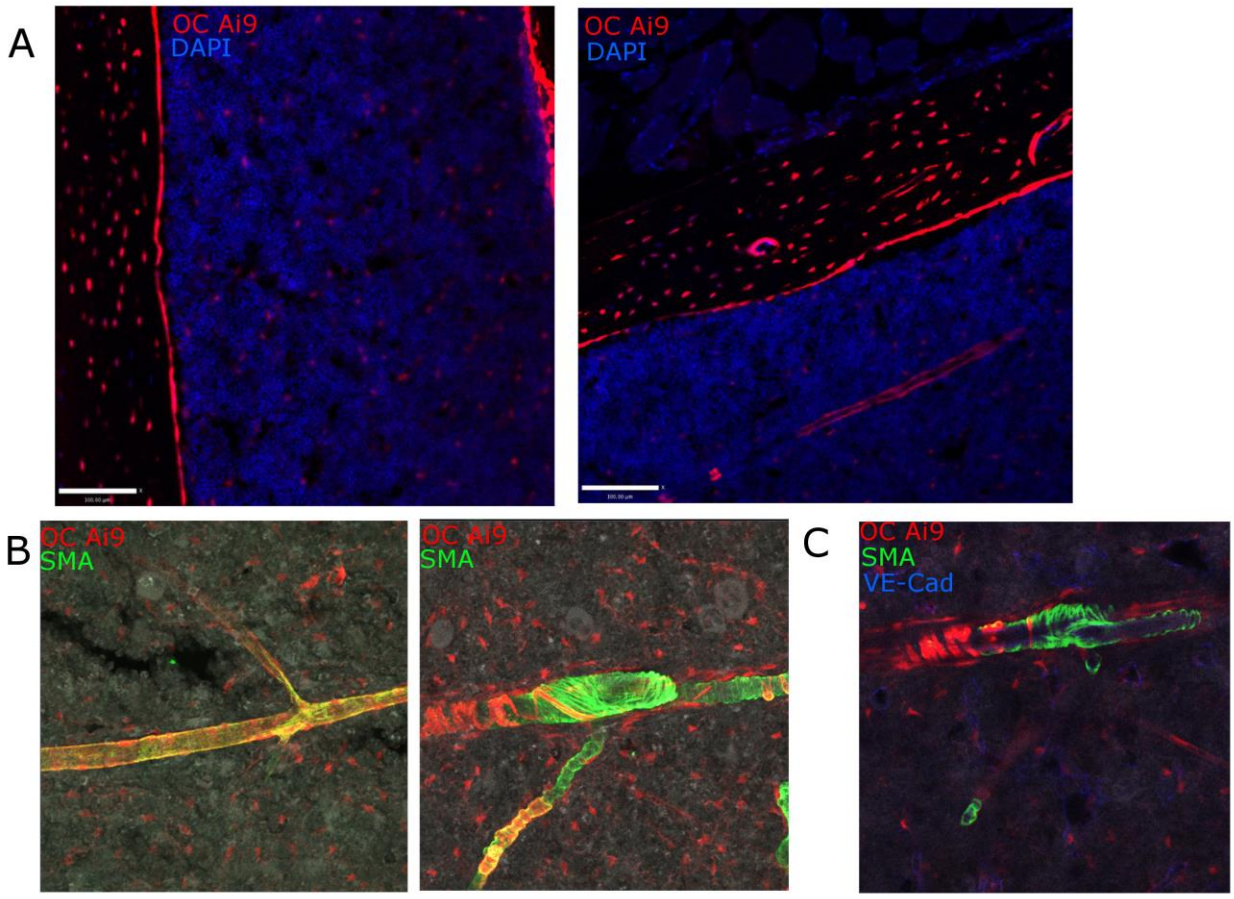

Figure 3.5. OC-Cre targets osteocytes, osteoblasts, central marrow cells, and periarteriolar smooth muscle cells. **A.** Representative photomicrographs of *OC-Cre Ai9* lineage mapping mice showing cells targeted by *OC-Cre* (red). DAPI is blue. **B.** Representative photomicrographs showing colocalization of *OC-Cre*-targeting (red) and smooth muscle actin staining (green). **C.** Representative photomicrograph showing *OC-Cre*-targeting (red), smooth muscle actin staining (green), and endothelial staining (VE-Cadherin, blue). Scale bar: 100 µm

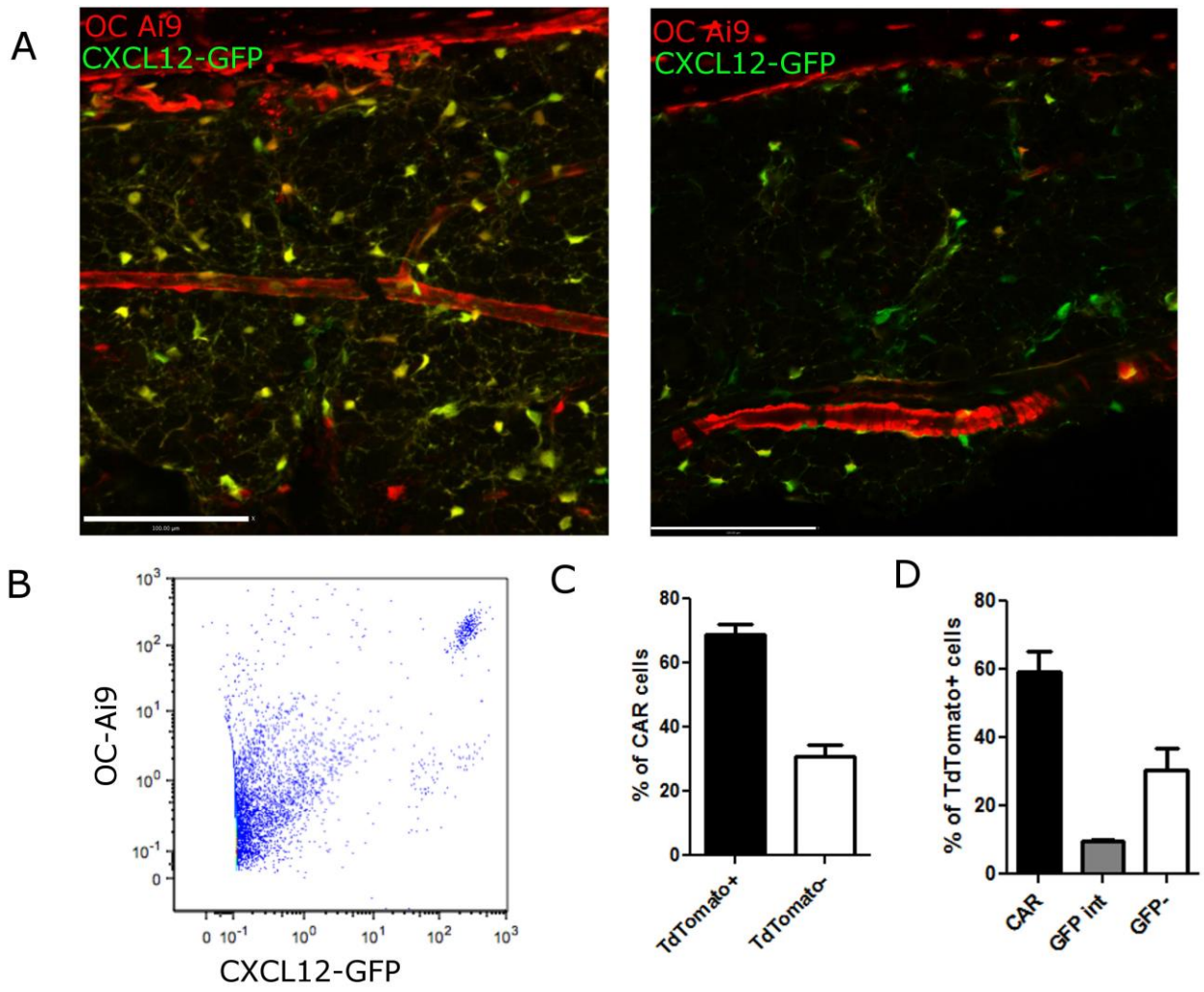

Figure 3.6. OC-Cre targets a subpopulation of CAR cells. **A.** Representative photomicrographs of *OC-Cre Ai9 Cxcl12-Gfp* lineage mapping mice showing cells targeted by *OC-Cre* (red) and expressing *Cxcl12-Gfp* (green). Scale bar: 100 µm. **B.** Representative flow cytometry plot showing $CXCL12^{GFP}$ expression (x axis) and *OC-Cre* targeting (y axis). Cells were gated on CD45- Ter119- CD31-. **C.** Quantification of the number of *OC-Cre*-targeted CAR cells (left panel) and frequency of CAR cells within the *OC-Cre*-targeted population (right panel). Data represent the mean ± SEM of 2 mice.

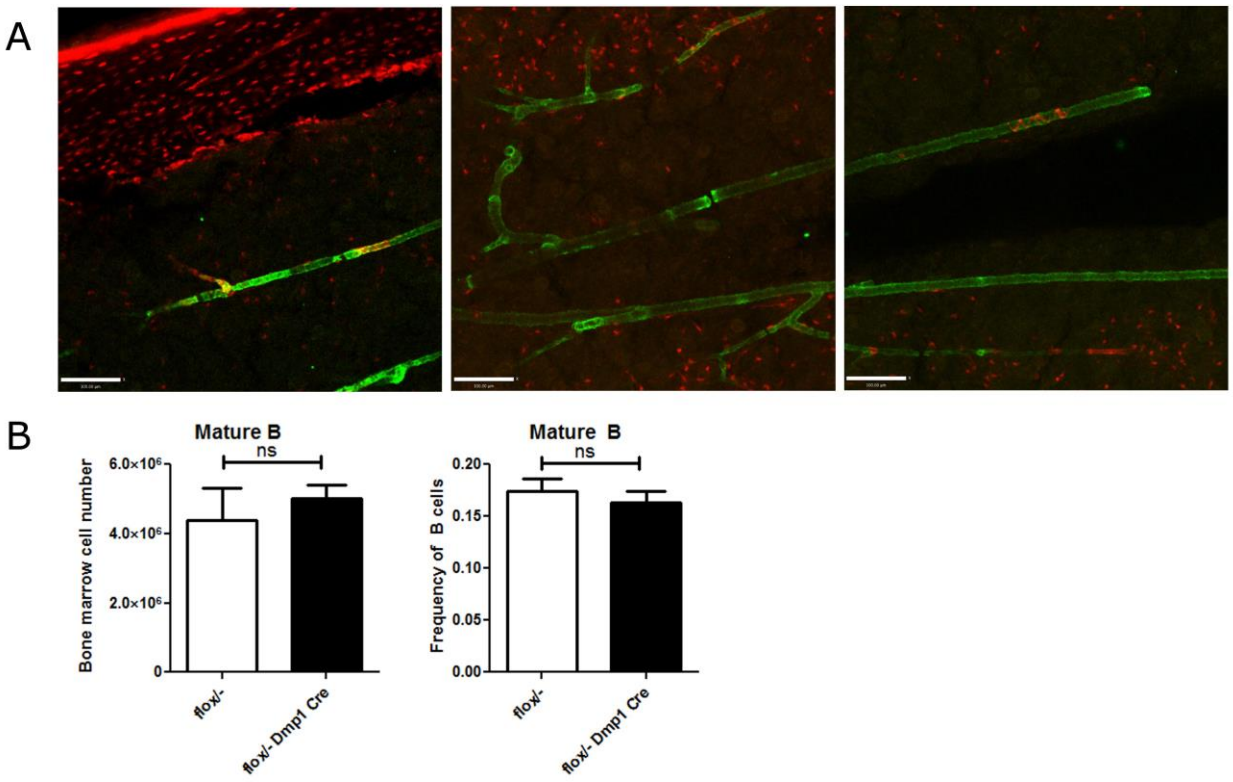

Figure 3.7. CXCL12 expression is osteocytes and osteoblasts is not required for mature B cell homing. **A.** Representative photomicrographs of *Dmp1-Cre Ai9* lineage mapping mice showing cells targeted by *Dmp1-Cre* (red) and expressing smooth muscle actin (green). Scale bar: 100 µm. **B.** Quantification of bone marrow mature B cell number (left panel) and frequency (right panel) in *Cxcl12*$^{fl/-}$ or *Dmp1-Cre Cxcl12*$^{fl/-}$ mice. Data represent the mean ± SEM of 5-13 mice. *P < 0.05; **P < 0.01; ***P < 0.001; ns: non-significant.

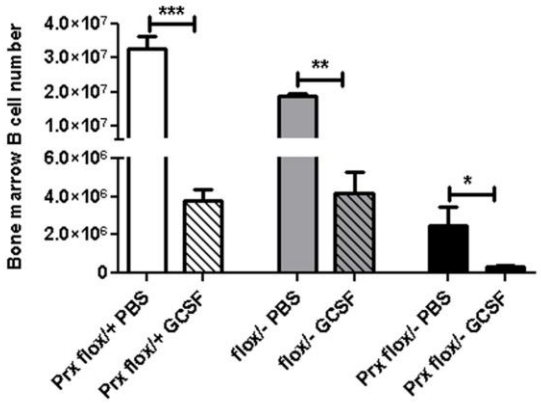

Figure 3.8. G-CSF-induced B cell suppression occurs, in part, via CXCL12-independent pathways. Mice of the indicated genotype were treated for 5 days with G-CSF or PBS, and the number of bone marrow B cells was quantified by flow cytometry. Data represent the mean ± SEM of 3-19 mice. *P < 0.05; **P < 0.01; ***P < 0.001; ns: non-significant.

CHAPTER 4: SUMMARY AND FUTURE DIRECTIONS

The primary goal of this work was to understand how the stromal bone marrow microenvironment regulates B lymphopoiesis. We took two approaches to examine the interactions between developing B cells and stromal cells. In Chapter 2, we examined how G-CSF regulates bone marrow stromal cells to decrease bone marrow B cell number. In Chapter 3, we used a *Cxcl12* conditional deletion model to dissect the role CXCL12 provided from different stromal cell populations plays in B lymphopoiesis. These questions have potential clinical and basic science relevance. Regulation of stromal cell function, the lineal relationship between different stromal cells, and how stromal cells interact with different hematopoietic populations in the bone marrow are poorly understood, and clarification provides insight into basic biology. The stromal niche has also been implicated in the maintenance of malignant cells in the bone marrow, and understanding how to modulate the supportive signals provided by stromal cells may point to new therapeutic regimens.

4.1. G-CSF reprograms the bone marrow microenvironment to suppress B lymphopoiesis

4.1.1. Summary

In Chapter 2, we explored how G-CSF affects stromal cells to suppress bone marrow B cell development. We showed that G-CSF works through cells of the monocyte-macrophage lineage to reprogram CAR cells, suppressing their adipogenic potential while downregulating their expression of B trophic factors. We also show that G-CSF decreases the number of mature osteoblasts in the bone marrow and downregulates the expression of B trophic factors in the remaining osteoblasts. These cumulative effect of these changes is severely impaired B lymphopoiesis. B cell number is decreased at all stages of bone marrow development, from the

BLP stage to the mature naïve B cell stage. The loss of B cells in the bone marrow is attributed in part to mobilization of early fraction B cells to the spleen where they undergo high rates of apoptosis, and in part to apoptosis in the bone marrow directly.

4.1.2. Can G-CSF synergize with chemotherapy in a mouse model of B-ALL?

Several lines of evidence indicate that the bone marrow microenvironment can shelter malignant cells from conventional chemotherapy. Niche factors have been reported to promote quiescence, associated with chemoresistance, in B-ALL cells[119,122]. Our results indicate that G-CSF promotes B cell cycling, and if G-CSF similarly induces cycling in malignant cells, it may be able to chemosensitize malignant blasts. The CXCL12-CXCR4 inhibitor AMD3100 has been shown to synergize with conventional chemotherapy in mouse models of B-ALL[120]. G-CSF disrupts B lymphopoiesis in part by downregulating CXCL12 expression in CAR cells and osteoblasts, suggesting that G-CSF may also be able to chemosensitize malignant cells. G-CSF affects multiple other B trophic factors besides CXCL12, including KIT ligand, IL-6, IL-7, FLT3L, BAFF, and IGF-1. Because G-CSF targets a broader range of B trophic factors, it may provide a more effective tool than the more narrowly-targeted AMD3100. To test this hypothesis, we have an ongoing study in which a B-ALL cell line engineered to express luciferase is injected into immunocompromised mice. After engraftment, mice are treated with chemotherapy, with or without G-CSF. Survival and tumor burden, as assessed by luciferase signal, are assessed over time. Correlative studies for cell cycling, apoptosis, and mobilization of blasts could also be performed to provide additional mechanistic insight. Future studies could involve the use of primary patient samples to determine if certain tumor profiles are more likely to be sensitive to G-CSF treatment.

4.1.3. What effect does G-CSF have on α-smooth muscle actin-positive pericytes?

Periarteriolar α-smooth muscle actin pericytes have recently been shown to be an important stromal cell population modulating hematopoietic stem cell quiescence[85]. Evidence from our *Cxcl12* conditional deletion model (Chapter 3) suggests that these pericytes may also play a role in B cell maintenance in the bone marrow, specifically the homing or retention of mature naïve B cells in the bone marrow. G-CSF treatment decreases mature naïve B cell number in the bone marrow. In part, this is due to increased mature B cell apoptosis, likely due to G-CSF-induced loss of dendritic cells in the bone marrow. Homing studies also indicate that mature naïve B cells do not home to the bone marrow of G-CSF-treated mice, similar to the phenotype observed in *OC-Cre Cxcl12*$^{flox/-}$ mice. Of note, expression of the *E-mu-Bcl2* transgene fails to rescue mature naïve B cell number in the bone marrow of G-CSF-treated mice. These data suggest that G-CSF treatment also results in an inability of mature naïve B cells to home or be retained in the bone marrow. Whether G-CSF has any effect on pericytes is not currently known, however. To examine the effect of G-CSF on pericytes, RNA expression profiling of pericytes from PBS versus G-CSF-treated mice could be performed. Identification of pericytes by flow cytometry is currently challenging. Anti-α-smooth muscle actin antibodies exist but require cell fixation and permeabilization which could complicate RNA extraction and yield. Endogenous reporters may provide an easier way to label, count, and sort pericytes. We have attempted to use *Myh11-Cre*, a Cre recombinase driven by the smooth muscle myosin light chain promoter, bred into a lineage mapping strain to identify α-smooth muscle actin-expressing pericytes in the bone marrow. Despite efficiently labeling smooth muscle cells, including vascular smooth muscle cells, in other organs (Figure 4.1A), *Myh11-Cre* failed to consistently or

predictably target bone marrow pericytes (Figure 4.1B). We have obtained *Transgelin-Cre* mice, expressing a Cre recombinase reported to be expressed in vascular smooth muscle cells. While expression of *Transgelin-Cre* in bone marrow vascular smooth cells has not been reported, a recent report has shown expression of Transgelin protein in bone marrow vascular smooth muscle pericytes[71]. Since the *Transgelin-Cre* is knocked-in to the endogenous *Transgelin* locus, expression of the Cre recombinases should faithfully reflect expression of the protein. Alternatively, *αSma-Gfp* reporter mice exist[84] and could also be used to label and sort pericytes with or without G-CSF exposure.

4.1.4. How does G-CSF treatment change CAR cell number and potential?

In our study, we observed an increase in CAR cell number. It is not currently known if CAR cells themselves are proliferating or if MSCs are proliferating to increase CAR cell number. Likewise, it is not known if the shift towards osteogenic differentiation in CAR cells is due to existing multipotent CAR cells differentiating down an osteogenic path, or if MSCs are producing CAR cells already committed to an osteoblastic fate. Distinguishing between these two possibilities would help to clarify how G-CSF exerts its effects on stromal cells and could point towards a role for G-CSF in MSC biology. These questions could be addressed in part by cell cycle analysis of MSCs and CAR cells in PBS versus G-CSF-treated mice. While MSCs are very rare and cell surface phenotypes are controversial, multiple mice could be pooled and different surface phenotypes could be analyzed to assess the cycling status of MSCs after G-CSF treatment. If G-CSF acts on MSCs, we would expect to see increased MSC cycling after G-CSF treatment. The effect of G-CSF on CAR cell proliferation could be similarly analyzed. The effect of G-CSF on the osteoblastic/adipogenic potential of MSCs could be tested by sorting MSCs

from G-CSF-treated mice and performing CFU-F assays to analyze the osteogenic and adipogenic activity of their progeny.

In G-CSF-treated mice, CAR cell number and osteogenic potential increases while mature osteoblast number decreases. The increase in osteogenic CAR cells post-G-CSF treatment could be a compensatory change due to this loss of osteoblasts, or it could be a direct consequence of G-CSF signaling through monocytic cells. An osteoblast ablation model, such as the *Col2.3-Tk* or *Dmp1-Cre-iDTR*, could be used to ablate osteoblasts in the absence of G-CSF treatment to determine if a similar increase in osteogenic CAR cells is observed. The alterations in hematopoiesis produced by osteoblast ablation[81] may make such a model difficult to interpret, however. An alternative approach would be to use a model overexpressing anti-apoptotic BCL2 in the osteoblast compartment. These models have their own drawbacks, however, including impaired osteoblast differentiation and osteocyte function[139].

4.1.5. How are CAR cells related to other reported stromal B niche populations?

CAR cells, osteoblasts, IL-7-expressing stromal cells, and Galectin-1-expressing stromal cells have all been reported to be niche cells for bone marrow B cells. While some reports have suggested that CAR cells, IL-7-expressing cells[63], and Galectin-1-expressing cells[64] are three distinct stromal cell populations, the evidence presented thus far is not entirely convincing, and by RNA expression CAR cells produce high levels of IL-7 and Galectin-1. It is possible that all three cell populations are distinct, that all three cell populations are the same, or that there is some overlap between the three. Recently developed tools allow the relationship between CAR cells and IL-7-expressing stromal cells to be clearly defined. An *Il7-Gfp* knock-in mouse strain[91,92] should allow accurate visualization of IL-7 expression, overcoming the weaknesses of

transgenic IL-7 reporter strains that contain fragments of the *Il7* promoter and are randomly inserted into the genome. While the *Il7-Gfp* allele could not be distinguished from *Cxcl12-Gfp*, an alternate readout of CXCL12 expression could be used, such as *Cxcl12-Dsred.* Alternatively, the only transgenic IL-7 reporter mouse reported to have bone marrow expression, *Il7-Cfp*[88], could be bred into the *Cxcl12-Gfp* background to distinguish the two cells provided endogenous CFP expression is sufficient for imaging. Antibody staining for Galectin-1 would allow quantification of the number of Galectin-1-expressing stromal cells that overlaps with CAR cells and IL-7-expressing cells to clearly define the relationship between these cell types.

B cells in the bone marrow have been reported to interact with different stromal cells at different stages of B cell development, with pre-pro-B cells interacting with CAR cells, pro-B cells interacting with IL-7-expressing cells[63], and early pre-B cells interacting with Galectin-1 cells[64]. One model of B cell development in the bone marrow suggests that a developing B cell moves from one cell type to another as the B cell matures. If CAR cells, IL-7-expressing cells, and Galectin-1 cells substantially overlap, it would provide evidence for a different model, that a single stromal cell is capable of providing a niche for multiple stages of B cell development. Such a model could get additional support from rigorously describing the relationship of different B cell subpopulations to the stromal cell populations identified. Our immunofluorescence assays are currently limited to 4 colors, restricting the number of markers that can be used for a single image and thus the number of cell populations that can be identified. We have successfully performed immunofluorescence for mature naïve B cells. Protocols for pre-pro-B cells and pro-B cells have been reported in the literature[63], but we have not been able to successfully reproduce these results. Additional work to develop robust staining panels for bone marrow B cell subsets will be necessary to describe the relationship of bone marrow B cells

to stromal cells. Alternatively, other groups have performed adoptive transfers using sorted cells to ask similar questions[64], though these results are potentially confounded by sorting, bone marrow homing, competition with similar cells in an unirradiated host, and a radically altered microenvironment in an irradiated host.

4.2. CXCL12 from stromal cell populations regulates B cell development

4.2.1. Summary

In Chapter 3, we explored the role that CXCL12 from different stromal cell populations plays in B cell development. Deletion of *Cxcl12* in mesenchymal stem and progenitor cells using *Prx1-Cre* resulted in almost total loss of bone marrow CXCL12 and a severe decrease in B cell number beginning at the CLP stage of lymphopoiesis. Deletion of *Cxcl12* in CAR cells using *Osx-Cre* resulted in a major reduction in bone marrow CXCL12 and a defect in B cell development beginning at the pre-pro-B stage. Deletion of *Cxcl12* in osteolineage cells using *OC-Cre* resulted in a minor change in total bone marrow CXCL12 level and decreased mature naïve B cells in the bone marrow due to a homing or retention defect. Since *Dmp1-Cre* or *Tie2-Cre*-mediated deletion of *Cxcl12* produced no phenotype, we concluded that mature osteoblast or endothelial-derived CXCL12 is dispensable for baseline B lymphopoiesis.

4.2.2. Is the decrease in mature naïve B cell number in *OC-Cre Cxcl12$^{flox/-}$* mice due to decreased homing or decreased retention?

Using an adoptive transfer model, we showed that fewer mature naïve B cells injected into the peripheral blood can be detected in the bone marrow of *OC-Cre Cxcl12$^{flox/-}$* animals relative to control animals 24 hours post-injection. This decrease could be due to impaired

homing from the peripheral blood/spleen to the bone marrow, or reduced retention of cells in the bone marrow. To distinguish between these two possibilities, we are planning to do *in vivo* imaging of our adoptive transfer model in collaboration with the Sipkins lab at Duke University. By imaging in real time at an early time point after the adoptive transfer, we will be able to determine how mature naïve B cells in the peripheral blood interact with the bone marrow. If decreased mature B cell number is due to decreased homing, we expect to see fewer mature naïve B cell exiting the peripheral blood and entering the bone marrow. If decreased retention is responsible, mature B cells should transit into the bone marrow at similar rates in *OC-Cre* *Cxcl12*$^{flox/-}$ and control mice but should not be retained in the bone marrow at the same rate. The same question could also be addressed by repeating the adoptive transfer experiment and sacrificing mice at various times prior to 24 hours.

4.2.3. What is the *OC-Cre*-targeted cell population that produces the CXCL12 required for normal mature naïve B cell homing/retention?

OC-Cre targets multiple different stromal cell populations in the bone marrow, but it is unclear which population is responsible for the loss of mature B cells in the bone marrow. By immunofluorescence, we observed targeting of osteocytes, osteoblasts, central marrow cells, and periarteriolar α-smooth muscle cells. *Dmp1-Cre* also targets osteoblasts and osteocytes, and since there is no loss of mature B cells in *Dmp1-Cre Cxcl12*$^{flox/-}$ mice, osteocytes and osteoblasts are unlikely to be responsible for producing CXCL12 required for mature B maintenance in the bone marrow. Both *OC-Cre* and *Dmp1-Cre* target a central marrow population. Flow cytometry in an *OC-Cre Ai9 Cxcl12-Gfp* reporter mouse shows that the majority of these cells are CAR cells, though notably there are untargeted CAR cells and targeted cells that are GFP^{intermediate} as

well. It is unknown if the central marrow cells targeted by *Dmp1-Cre* are also CAR cells, or if the extent of targeting is the same. *Dmp1-Cre Ai9 Cxcl12-Gfp* mice could be generated and compared to *OC-Cre Ai9 Cxcl12-Gfp* mice to determine if the populations are similar. If both *Dmp1-Cre* and *OC-Cre* do target similar populations to the same extent, these central marrow cells are also unlikely to be responsible for the mature B cell deficit. *Lepr-Cre* may also be useful in resolving this question. *Lepr-Cre* targets all CAR cells[71,73], and if *Lepr-Cre Cxcl12*$^{flox/-}$ mice show no defect in bone marrow mature B cell number, CAR cells can be excluded as candidates.

Periarteriolar smooth muscle cells are targeted extensively by *OC-Cre* but only rarely targeted by *Dmp1-Cre* and thus represent an attractive candidate for mediating the mature B cell defect observed in *OC-Cre Cxcl12*$^{flox/-}$ mice. Our first attempt to target these cells using a Cre recombinase driven by the smooth muscle myosin light chain promoter (*Myh11-Cre*) failed to target these cells in the bone marrow (Figure 4.1). We have begun breeding a second smooth muscle Cre line, *Transgelin-Cre*, into our lineage mapping and *Cxcl12*^{flox} colonies to determine if *Transgelin-Cre* will target our cells of interest. Because Transgelin protein expression has been described in bone marrow periarteriolar smooth muscle cells[71] and the *Transgelin-Cre* is a knock-in into the endogenous *Transgelin* locus, we expect to see appropriate targeting. If the *Transgelin-Cre* does not target appropriately, however, there are additional alternatives, including both constitutive and inducible *α-Smooth Muscle Actin-Cre* lines[140,141] and the *Ng2-Cre* line[85]. Because periarteriolar smooth muscle cells may be osteoprogenitors[84], *Transgelin-Cre* may target both smooth muscle cells and osteoblasts, making it difficult to prove that *Cxcl12* deletion in periarteriolar smooth muscles alone, rather than the combination of loss in periarteriolar smooth muscle cells and osteoblasts, is responsible for the loss of mature B cells. It is also possible that the periarteriolar smooth muscle cell compartment contains MSCs, in which

case targeting of CAR cells and other stromal cells may also be observed. If *Transgelin-Cre* does target broadly, a *Transgelin-DTR* line[142], allowing ablation of only cells currently expressing Transgelin, has been reported and may be useful in providing correlative evidence for a specific role of periarteriolar smooth muscle cells in mature B cell homing/retention.

Localization of mature B cells in the bone marrow may also help identify the cell type that produces the CXCL12 required for mature B cell maintenance. While mature B cells have been reported to be localized perivascularly and near dendritic cells[93], their relationship with bone marrow stromal cells has not been described. Localization of adoptively transferred mature naïve B cells into an *OC-Cre Ai9 Cxcl12-Gfp* mouse may show a relationship to a particular subset of *OC-Cre*-targeted cells, providing suggestive evidence of a role for that cell type in mature B cell maintenance in the bone marrow.

4.2.4. What are the central marrow cells targeted by *OC-Cre*?

Because stromal cells in the bone marrow are currently poorly defined, dissecting the nature of the cells targeted by *OC-Cre* may be useful in better understanding the stromal microenvironment. Approximately 40% of the *OC-Cre*-targeted cells in crushed bone marrow are not CAR cells, and it is unknown what they represent. They could include periarteriolar smooth muscle cells, in which case co-staining with an antibody against α-smooth muscle actin or NG2 should produce costaining. They also may represent a stage of osteoblast development. Costaining for an osteoblast marker such as ALCAM or Osteocalcin could be informative, and sorting followed by CFU-F or CFU-OB culture could be used to demonstrate osteogenic activity. Alternatively, an unbiased approach like gene expression profiling via gene chip or RNA-seq

could be performed to probe the gene expression signatures of these different populations for clues about their nature.

OC-Cre targets approximately two-thirds of CAR cells in the crushed bone marrow, leaving one-third untargeted. In the literature, CAR cells have been described as a homogenous population since the surface markers and transcripts examined thus far seem to be expressed at similar levels in all CAR cells[74]. Our *OC-Cre* targeting data thus represent a heterogeneity in CAR cells that has not been previously reported. Because *OC-Cre* is typically considered an osteolineage marker, *OC-Cre* targeting within CAR cells may represent commitment to the osteoblast lineage. Sorting followed CFU-F, CFU-OB, and CFU-adipocyte assays could be used to test this hypothesis, as could flow cytometry for osteolineage markers. Sorting followed by RNA expression analysis could also be used to examine the extent to which targeted CAR cells vary from non-targeted CAR cells in their expression of adipogenic and osteogenic genes. *OC-Cre Ai9 Cxcl12-Gfp* mice could also be treated with G-CSF to determine if CAR cell number is increased in the targeted compartment, the untargeted compartment, or both.

4.2.5. Are other non-malignant or malignant B cell populations affected by *Cxcl12* deletion in specific stromal cells?

CXCL12-CXCR4 signaling is known to play an important role in the homing and engraftment of B cell populations not examined in our study. Both plasmablasts and plasma cells require CXCR4 in order to home to the bone marrow, at least at early time points[20]. The homing and engraftment of malignant B cells is also regulated by CXCL12[31,120]. Examining the number of plasmablasts and plasma cells in the bone marrow of conditional deletion mice, under baseline conditions or following immunization, could provide insight into the cells responsible for

mediating the maintenance of these cells in the bone marrow. Analysis serum immunoglobulin level, both pre- and post-challenge, could reveal a defect in humoral immunity. Adoptive transfer of B-ALL, CLL, or lymphoplasmacytic lymphoma cell lines or primary cells into conditional deletion mice could reveal which compartment, if any, is specifically responsible for recruiting malignant B cells to the bone marrow.

4.2.6. Is niche-specific expression of CXCL12 required for normal B lymphopoiesis, or is total bulk level sufficient?

Our data indicate that deletion of *Cxcl12* within different stromal cells results in impaired B lymphopoiesis at different stages of development. Because the stromal cells we analyzed are lineally related, however, our data also show progressive drops in total bone marrow CXCL12 level that correlate with phenotype severity. Two different models could explain our results. In the first model, CXCL12 must be provided from a specific stromal cell population to a B cell at a specific development stage. In this model, MSCs provide CXCL12 required for CLP development. CXCL12 from CAR cells is required for pre-pro-B cells but not for CLPs, while *OC-Cre*-targeted cells provide CXCL12 required for mature B cells but not earlier stages of B lymphopoiesis. Because the B cell must be located in the proper niche in order to continue to develop normally, CXCL12 from a different source cannot substitute for CXCL12 from the appropriate niche cell. In the second model, different stages of B cells are differentially sensitive to the total level of CXCL12 in the bone marrow. CLPs require only very low levels of total CXCL12, and thus are affected by deletion of *Cxcl12* in MSCs, which totally ablates CXCL12 expression in the bone marrow, but not by other Cre models which have higher levels of remaining CXCL12. Pre-pro-B cells require intermediate levels of CXCL12, while mature B

cells are sensitive to even minor perturbations in total CXCL12 level. Understanding which of these two models is correct is important since the idea of a "niche" is generally assumed to require cell-cell contact. If CXCL12 from any bone marrow source is capable of regulating B cell development as well as CXCL12 from a specific stromal cell, the commonly held view of CXCL12 as a "niche molecule" in B lymphopoiesis may not be accurate.

The different models can be distinguished in two different ways. In the niche-specific model, developing B cells would be expected to be physically near their putative niche cell. Consistent with this model, pre-pro-B cells have been reported to be in contact with CAR cells[63]. CLPs have been reported to be in contact with osteoblasts[73], but their relationship to MSCs has not been described. Likewise, mature B cells have been reported to be in perivascular dendritic cell niches[93], but their relationship to *OC-Cre*-targeted cells has not been reported. The second way to distinguish between the two models is via a CXCL12 rescue experiment. Restoring bulk levels of CXCL12 in a conditional deletion mouse, but having the CXCL12 come from a different cell source than the cell type targeted by the deletion, would be expected to rescue the B cell defect in model 2 but not model 1. We attempted to perform a rescue experiment by transplanting hematopoietic cells infected with a CXCL12-overexpressing lentivirus into *Prx1-Cre Cxcl12*$^{flox/-}$ recipients. However, we were unable to sufficiently restore total CXCL12 levels, in part due to selection against CXCL12-infected hematopoietic cells relative to empty vector-infected cells (Figure 4.2). One alternate approach could include using an inducible virus system to reduce selection against CXCL12-infected hematopoietic cells. Alternatively, a transgenic mouse model could be generated, restoring CXCL12 expression in CAR cells or osteoblasts on a *Prx1-Cre Cxcl12*$^{flox/-}$ background, for example. Such a model could even be inducible. However, no promoters have yet been identified that would be appropriate to drive high-level CXCL12 in a

specific stromal cell population, and generation of the transgenic mice would be a time-consuming and expensive endeavor.

4.3. Conclusion

In Chapter 2 of this thesis, we described how G-CSF suppresses B cell development in the bone marrow. We show that G-CSF signals through monocyte lineage cells to alter the expression of B trophic factors in supportive stromal cell populations, resulting in B cell mobilization and apoptosis. We also show that G-CSF alters CAR cell potential, promoting osteogenic differentiation over adipogenic differentiation. In Chapter 3 we explored the role CXCL12 from different stromal cell populations plays in B lymphopoiesis. While CXCL12 from endothelial cells and osteoblasts/osteocytes appears to be dispensable for baseline B lymphopoiesis, CXCL12 from MSCs is required for CLP maintenance, CXCL12 from CAR cells is required for pre-pro-B maintenance, and CXCL12 from *OC-Cre*-targeted cells is required for mature B cell maintenance. These data help clarify how stromal cells interact with developing B cell in the bone marrow, and how an exogenous factor can be used to alter hematopoiesis by inducing microenvironmental changes. Though our studies were limited to non-malignant B cells and limited stromal cell subsets, the results generated may provide insight into the broader stromal cell microenvironment and may provide insights that are therapeutically useful in treating B cell malignancies.

4.4. FIGURES

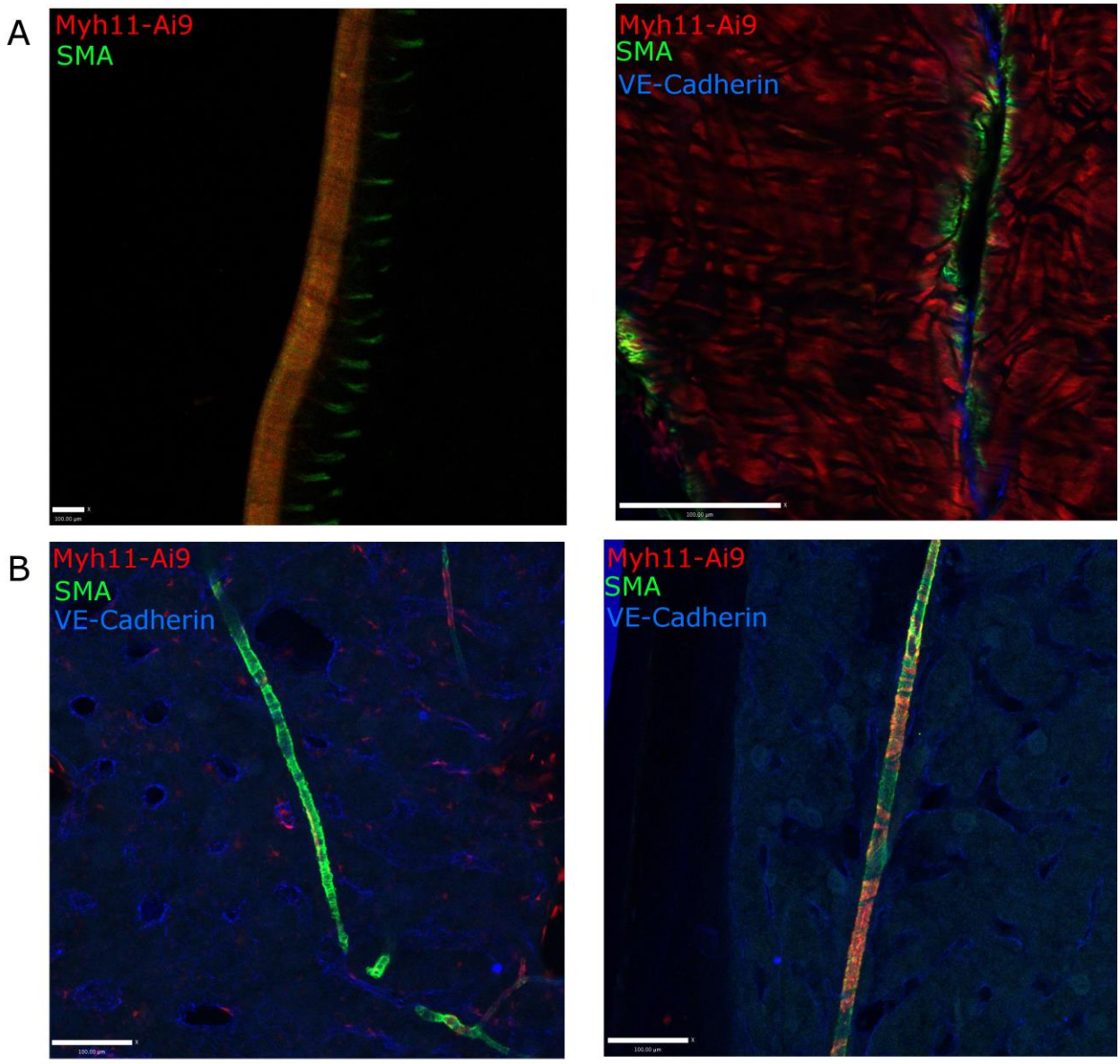

Figure 4.1. Myh11-Cre does not consistently target vascular smooth muscle in the bone marrow. **A.** Representative photomicrographs showing *Myh11-Cre*-targeted cells (red) and smooth muscle actin staining (green) in small intestine (left panel) and aorta (right panel). **B.** Representative photomicrographs showing *Myh11-Cre*-targeted cells (red) and smooth muscle actin staining (green) in bone marrow. Scale bar: 100 µm.

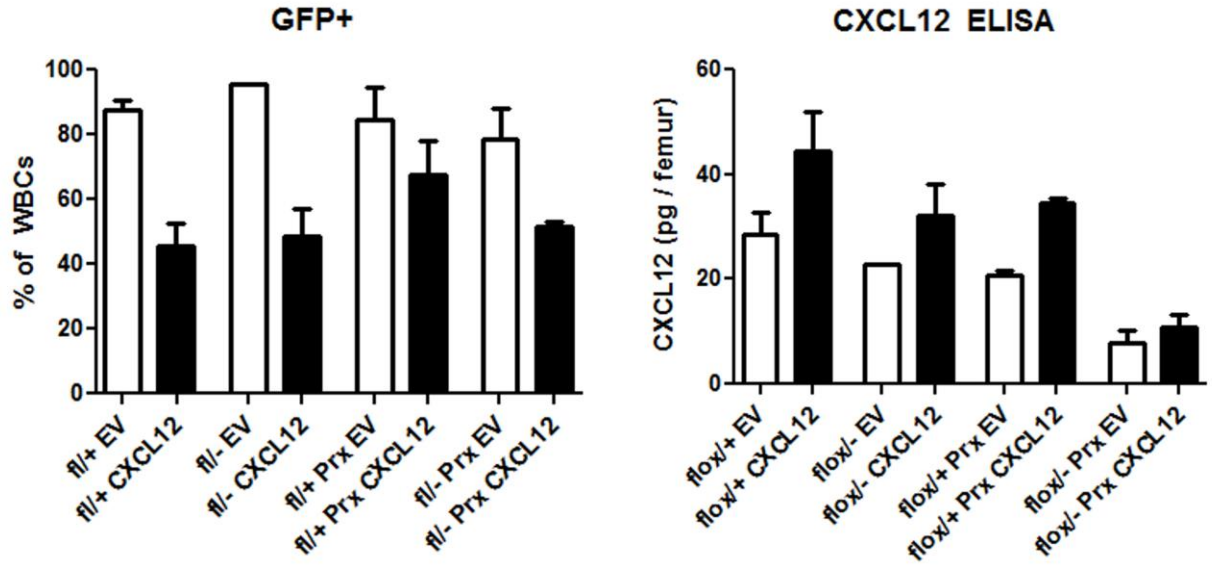

Figure 4.2. Lentiviral expression of CXCL12 in hematopoietic cells is not sufficient to rescue bulk bone marrow CXCL12. **A.** GFP chimerism corresponding to lentivirally-transduced cells in the peripheral blood of transplanted mice of the indicated genotype and viral infection 6 weeks post-transplant. **B.** CXCL12 ELISA showing the amount of CXCL12 protein in the bone marrow of mice of the indicated genotype transplanted with hematopoietic cells of the indicated virus infection.

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